Annals
of the
Missouri Botanical Garden

VOL. 6 SEPTEMBER, 1919 No. 3

# AN EDIBLE GARDEN HEBELOMA[1]

EDWARD ANGUS BURT

*Mycologist and Librarian to the Missouri Botanical Garden*
*Professor in the Henry Shaw School of Botany of Washington University*

The species of *Hebeloma* have been so invariably found in woods in autumn in the past that it occasioned great surprise to find a fine species of this genus in great abundance, June 3, in cultivated borders of the Missouri Botanical Garden. The mycelium of this species was well developed and could be followed to the strawy manure which had been spaded underground in the borders.

Species of *Hebeloma* have usually a nauseous odor or taste, or an odor or taste of radishes, or may be bitter, and are regarded as unwholesome and, in some cases, even poisonous. Specimens of this garden collection were of good size, with pileus 4–10 cm. in diameter, not infested with larvae, not rapidly putrescent, with a pleasant farinaceous taste and odor. This species gave promise of being a very desirable acquisition if its edibility could be established. This was done by cooking specimens in butter and eating a small portion of a pileus with other food at dinner. As no disagreeable symptoms were experienced over night, double the quantity of the fungus was eaten at breakfast. This procedure was carried on until three fructifications were eaten at one time, when others also ate the fungus with equal freedom and decided that this species is palatable and has a delicious characteristic flavor.

The specimens did not have as large size and as great weight

[1] Issued October 11, 1919.

as those of *Agaricus campestris* when the latter is grown as a mushroom for the market but they are of good size and weight, are firm and keep well, and have a flavor of good quality which is distinct from that of *Agaricus campestris*. These are desirable qualities in a market species of mushroom. The origin of the mycelium in the strawy manure which was spaded into the soil suggests that preparation of the spawn and method of growing under cultivation might be the same as those employed for the common mushroom, *A. campestris*.

This garden *Hebeloma* is apparently of local occurrence, for its characteristics do not agree with those of any species heretofore known. It may be that the normal season of this mushroom is spring or early summer, as is the case with *Pholiota vermiflua*, a species which was abundant in the Missouri Botanical Garden at the same time. The month of May preceding had been very wet and with frequent storms throughout the month. The name and description of this species are as follows:

**Hebeloma hortense** Burt, n. sp. Plate 3.

§*Denudata*. Pileus glabrous, veil absent from the first.

Type: in Mo. Bot. Gard. Herb., 54130, and in Burt Herb.

Pileus fleshy, convex, umbonate, becoming expanded, even, glabrous, pale Isabella-color when moist, becoming cartridge-buff, with the umbo pinkish buff, the margin slightly inrolled when very young; flesh whitish, farinaceous; odor not disagreeable, not of radishes; lamellae broad, slightly sinuate, moderately close, white at first, becoming Isabella-color, the edge entire and not distilling drops; stem fleshy, hollow, equal or slightly bulbous at the base, fibrillose, mealy above when young, with no trace of a veil or cortina; spores Rood's brown in spore collection, even, 10–11 x 6 $\mu$, borne in fours on protruding basidia; no cystidia present or not noteworthy.

Pileus 4–10 cm. broad; stem 4–9 cm. long, 4–10 mm. thick.

In cultivated borders where a straw manure had been worked into the ground. St. Louis, Missouri. Abundant in early June after prolonged rains.

The fructifications occur singly or in small clusters of two or three. This species is noteworthy in its genus by absence of

viscidity and odor of radishes, and by its large size and occurrence in abundance in cultivated ground. Nearly all other species of *Hebeloma* are inhabitants of forests and occur there sparingly. Fresh specimens have a pleasant farinaceous taste and odor, and keep well. This species is edible and with a pleasant and distinctive flavor.

## EXPLANATION OF PLATE

### PLATE 3

The figures of this plate have been reproduced natural size.

A cluster of two specimens viewed from above to show form of pileus.

A single specimen showing the lamellae and stem.

Median vertical section through pileus and upper part of stem to show breadth and attachment of the lamellae.

Transverse section of the hollow stem.

A very young fructification split lengthwise to show slightly inrolled margin of pileus and the absence of a veil or cortina.

BURT—HEBELOMA HORTENSE

# PROTOMERULIUS FARLOWII BURT, N. SP.[1]

EDWARD A. BURT

*Mycologist and Librarian to the Missouri Botanical Garden*
*Professor in the Henry Shaw School of Botany of Washington University*

During his last illness and only two days before his death, Dr. Farlow had mailed to me a very interesting fungus which he collected near his summer home at Chocorua, New Hampshire. This fungus, which I have named *Protomerulius Farlowii*, has apparently more minute pores than have been recorded for any species heretofore described. The pores are so minute that to the naked eye the fructification has the aspect of a very thin *Sebacina* with its hymenial surface slightly pruinose. This pruinose surface becomes barely visible as irregularly angular and somewhat sinuous pores with very thin dissepiments when viewed through a pocket magnifier of fine definition having a magnification of 10 or more diameters, and is beautifully shown under the compound microscope with an objective of about 16 mm. focal distance.

Under this higher magnification the dissepiments appear as thin, irregular folds up to 30 $\mu$ high and about 20 $\mu$ thick, with the edge acute and lacerate. The angular pores are incompletely enclosed by the dissepiments; the hymenial configuration is that of *Merulius* rather than *Poria*.

Preparations of the hymenium show longitudinally cruciately septate basidia 9–10 $\times$ 7 $\mu$. Hence this fungus is a member of the *Tremellaceae* and has the hymenial configuration of a *Merulius*.

A. Möller collected at Blumenau, Brazil, a fungus having the form of a *Merulius* and longitudinally cruciately septate basidia, which he published[2] as *Protomerulius brasiliensis* new genus and species. Although the hymenial folds and pores are much smaller and less perfectly developed than those of *Protomerulius brasiliensis*, the generic description of *Protomerulius* applies well to the New Hampshire specimen.

Although 24 years have elapsed since the publication of *Proto-*

[1] Issued October 11, 1919.

[2] Bot. Mitt. a. d. Tropfen 7:60. 1895; 8:129, 172. *pl. 3. f. 3, 4, pl. 5. f. 36.*

*merulius* I fail to find record that collections referable to this genus or its single species have been made elsewhere in this rather long interval of active mycological exploration. It is therefore remarkable that the presumably tropical genus *Protomerulius* should have so noteworthy a species as *P. Farlowii* in northern New Hampshire at a rather high altitude.

The color of the specimens of *P. Farlowii* is noted as purple when in vegetative condition and suggestive in aspect of a species of *Tulasnella*, but this color was soon lost in drying and the specimens are now pale olive-gray of Ridgway. The fructifications occur on the surface of decayed coniferous wood, on the rough surface of which a slender foliaceous hepatic is present also.

Vertical sections through the fructification and substratum show the fructification to be a continuous compact membrane 10–15 μ thick; this membrane is composed of longitudinally arranged, thin-walled, hyaline hyphae crowded closely together. Branches from the hyphae of this membrane curve outward here and there and terminate in clusters of basidia. The basidia are somewhat interruptedly arranged in the hymenium rather than densely. At intervals of about 40 μ hyphae grow outward from the membrane to form the tramal tissue of the folds or dissepiments. These folds are about 30 μ high and 20 μ thick and covered by the hymenium. The membranous layer of the fructification is elevated about 40 μ above the surface of the wood and supported by groups of hyphae which arise from the substratum. These details are shown in the accompanying text-figures.

The formal description of this species is as follows:—

**Protomerulius Farlowii** Burt, n. sp.

Type: in Farlow Herb. and Mo. Bot. Gard. Herb.

Fructifications resupinate, effused, gelatinous, membranaceous, very thin and tender, separable with care when moist, "purple" when fresh, becoming pale olive-gray upon drying, pruinose to the naked eye, but showing under the microscope an imperfectly porose surface with thin, irregular folds and dissepiments more or less lacerate, the edges thin; pores angular-sinuose, about 40 μ in diameter or 25 to a mm.; in structure 20–30 μ thick, with a compact subhymenial layer 10–15 μ thick,

composed of densely and longitudinally arranged, hyaline, thin-walled hyphae 3 μ in diameter; subhymenial layer elevated above the substratum by scattered clusters of hyphae; basidia longitudinally cruciately septate, 9–10 × 7 μ, with slender sterigmata; spores hyaline, even, subglobose, 6 × 5 μ.

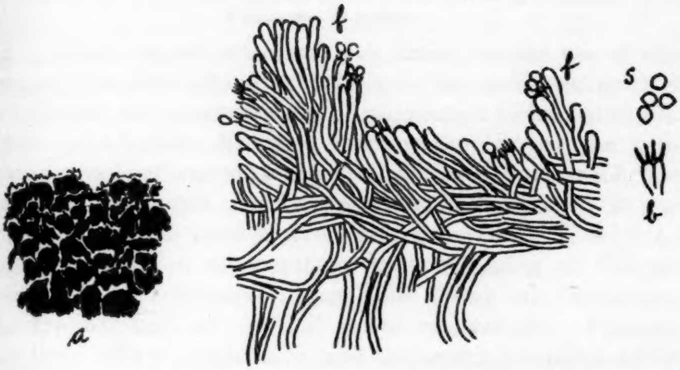



Fig. 1. *P. Farlowii. a*, fructification viewed from above, showing pores in black and folds and dissepiments in white, × 68; *f* and *f*, section of fructification showing pore between folds, × 375; *b*, basidium, and *s*, spores, × 375.

Fructifications in small gregarious patches 2–10 mm. in diameter.

On very rotten, decorticated, coniferous wood. New Hampshire. September. Probably very rare.

*P. Farlowii* should be recognized in the field by its purple color and aspect of *Tulasnella*, and the pruinose surface which is shown by a good lens to have the surface configuration of *Merulius*. The very minute, angular pores, thin and lacerate dissepiments with acute edges, the very small fructifications, and purple color when fresh separate this species from *P. brasiliensis*.

Specimens examined:

New Hampshire: Chocorua, *W. G. Farlow*, 6*, type (in Farlow Herb. and in Mo. Bot. Gard. Herb., 55596).

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

# THE MICRO-COLORIMETER IN THE INDICATOR METHOD OF HYDROGEN ION DETERMINATION

B. M. DUGGAR

*Physiologist to the Missouri Botanical Garden, in Charge of Graduate Laboratory*
*Professor of Plant Physiology in the Henry Shaw School of Botany of Washington University*

In a recent paper[1] attention was drawn to the use of the colorimeter in determining accurately by the indicator method the hydrogen ion concentration of pigmented biological fluids. In the method there discussed there was employed the well-known principle of compensating for the color of the test solution by introducing the test solution also as a shield solution. It was pointed out that in order to avoid the optical difficulties of the usual tintometers or comparators, while retaining all the advantages of the colorimeter, glass cells fitting one within another are arranged as cup and shield respectively. Properly made these cells are expensive, and the method requires about 15–20 cc. of the liquid for convenient determinations by the colorimeter.

Recently I have had occasion to test the hydrogen ion concentration of some fluids obtainable only in small quantity, and while examining the possibilities of adapting the micro-colorimeter for this work it became clear that the Dubosq type of this instrument lends itself admirably to the colorimetric method in general, and to small quantities of fluid in particular. Moreover, as the method is now modified the necessity for special cells is eliminated. Instead of employing two special cells in connection with each plunger of the colorimeter the principle of the new procedure lies in the use of the plunger tube and of a colorimeter cup as cells on each side of the system. When the quantity of test material available is not limited it is customary in our work to employ 5-cc. quantities, and 5-cc. quantities of the standards, the solutions being prepared in small serological test-tubes to each of which is added 3 drops of indicator. With

[1] Duggar, B. M., and Dodge, C. W. The use of the colorimeter in the indicator method of H ion determination with biological fluids. Ann. Mo. Bot. Gard. 6: 61–70. *f. 1.* 1919.

the micro-colorimeter a quantity as small as 1 cc. of sample may serve both for test solution and for the shield. It is preferable, however, to have not less than 2 cc. for most careful work.

The standardization of the apparatus for this work is extremely simple. It is merely necessary to know the volume of the plunger tube and its length so that in the determinations it will be possible to place a given volume of solution in the plunger cylinder, and knowing the depth which this will occupy, the instrument may be set so that a similar depth will be examined in the colorimeter cup. This is important, since in one case, as described later, the pigmented sample is placed in the plunger tube and in the other case in the colorimeter cup. Therefore equal depths of solutions will be examined in both cells of the systems. In the instrument at our disposal the plunger tube is 33 mm. in length and the volume 1.25 cc. Since the tube is cylindrical the volume is proportional to length, so that if .625 cc. of solution is added the column has a depth of 16.5 mm. The depth need not be so great as this, and 0.5 cc. of liquid is sufficient. If the quantity of the solution employed is reduced beyond this point, it is necessary to increase relatively the amount of indicator added. Where the total quantity of the test solution is 2 cc., 1 cc. being employed for the sample and 1 cc. for the shield, we find it desirable to use 1 drop of indicator for the 1-cc. sample. If this proves too highly colored the indicator may, of course, be diluted one-half.

I find it desirable to arrange the samples, standard, and shields as follows:

> In the left plunger tube place the measured quantity of plain water as shield, and in the colorimeter cup of that side place a quantity of the sample or test solution plus indicator which shall give any depth greater than that of the liquid column in the plunger tube. In the right plunger tube place the measured quantity of the sample as shield, and in the right colorimeter cup the standard solution plus indicator.

With this arrangement it is desirable to make up a few standards covering the range of probability, and then in making the determination it is only necessary to change the solution in the right colorimeter cup until an exact match is obtained. There

is one slight optical defect due to the fact that the surfaces of the liquids in the plunger tubes are not plane surfaces, but this is of no practical consequence in the actual determination, especially where a strong and standard source of light is employed. It is recommended that any of the so-called daylight bulbs be employed in this work. Using the method mentioned no difficulty whatever has been experienced in determining rapidly and effectively the hydrogen ion concentration of such dark liquids as oxidized potato juice, carrot decoction, and decoctions of plants containing considerable chlorophyll.

It is perhaps unnecessary to add that the technique suggested is equally applicable where the large types of colorimeters are employed, such as the Dubosq of standard size, or the Kober (if plunger tubes are detachable), but somewhat larger quantities of solutions will be required.

*Graduate Laboratory, Missouri Botanical Garden.*

# STUDIES IN THE PHYSIOLOGY OF THE FUNGI

## VIII. Mixed Cultures

S. M. ZELLER

*Formerly Visiting Fellow in the Henry Shaw School of Botany of Washington University*

and HENRY SCHMITZ

*Formerly Rufus J. Lackland Fellow in the Henry Shaw School of Botany of Washington University*

Some consideration has been given in previous literature to the behavior of fungi in mixed cultures, but this has been sufficiently reviewed by Harder,[1] whose work along the line seems to be the most complete. His conclusions, however, are based merely upon observations on the rate of growth and color production in the medium and mycelium. The purpose of his work was to determine whether the inhibition or stimulation of growth, as the case may be, might not be the result of the depletion of the available carbohydrates in the medium or a change in the hydrogen ion concentration.

In the present work the following fungi were used: *Lenzites vialis* Pk., *Merulius pinastri* (Fr.) Burt, *Daedalea quercina* (L.) Fr., *Trametes Peckii* Kalchbr., *Pleurotus sapidus* Kalchbr., *Merulius lacrymans* (Wulf.) Fr., *Lentinus lepideus* Fr., *Daedalea confragosa* (Bolt.) Fr., *Coniophora cerebella* Pers., *Polystictus versicolor* (L.) Fr., *Isaria* sp., *Polyporus lucidus* (Leys.) Fr., *Polystictus hirsutus* Fr., *Aspergillus glaucus*,[2] *A. niger* Van Tieg., *A. fumigatus* Brizi, *A. versicolor* Tiraboschi, and *A. Sydowi* Bainier and Sartory.

All the fungi were grown upon 2 per cent potato agar plates prepared in the manner previously described.[3] After growth

[1] Harder, R. Über das Verhalten von Basidiomyceten und Ascomyceten in Mischkulturen. Naturwiss. Zeitschr. f. Forst- u. Landw. 9: 129–160. *pl. 3–4. f. 1–2.* 1911.

[2] Thanks are due to Dr. Charles Thom for the determination of the *Fungi Imperfecti* included in this list.

[3] Zeller, S. M., Schmitz, H., and Duggar, B. M. Studies in the physiology of the fungi. VII. Growth of wood-destroying fungi on liquid media. Ann. Mo. Bot. Gard. 6: 137–142. 1919.

TABLE I

| Fungus | Organisms stimulating | Organisms inhibiting | Organisms overgrowing | Organisms not influencing |
|---|---|---|---|---|
| L. vialis | D. quercina* | T. Peckii*, P. sapidus*, Isaria*, A. niger†, A. Sydowi* | T. Peckii, P. versicolor, P. hirsutus | L. vialis, L. lepideus, D. confragosa |
| M. pinastri | | D. quercina†, T. Peckii*, L. lepideus*, D. confragosa†, C. cerebella†, P. versicolor*, P. lucidus*, P. hirsutus*, A. fumigatus†, A. Sydowi* | M. pinastri, T. Peckii, P. sapidus, A. niger | M. pinastri |
| D. quercina | M. lacrymans*, D. confragosa*, P. lucidus*, P. hirsutus* | M. pinastri†, L. lepideus*, C. cerebella*, Isaria*, A. niger* | T. Peckii, P. sapidus, P. versicolor | L. vialis, D. quercina |
| T. Peckii | D. quercina*, A. fumigatus*, A. glaucus* | D. confragosa*, Isaria*, A. glaucus*, A. versicolor†, A. Sydowi* | P. sapidus | |
| P. sapidus | | L. vialis*, L. lepideus†, P. versicolor†, P. lucidus*, P. hirsutus† | D. quercina, M. lacrymans | P. sapidus |
| M. lacrymans | | L. lepideus†, P. versicolor†, P. lucidus†, A. fumigatus† | M. pinastri, T. Peckii | |
| L. lepideus | | M. pinastri*, P. sapidus†, P. lucidus† | T. Peckii, P. versicolor | L. vialis |
| D. confragosa | M. lacrymans† | M. pinastri†, T. Peckii†, P. lucidus†, P. hirsutus† | P. sapidus | L. vialis, A. fumigatus |
| C. cerebella | L. vialis,* M. lacrymans† | M. pinastri†, D. quercina†, P. versicolor*, A. glaucus*, A. niger* | T. Peckii | |

| | | | | |
|---|---|---|---|---|
| P. versicolor | T. Peckii* | A. versicolor†, A. Sydowi†, M. pinastri*, P. sapidus†, A. niger*, M. lacrymans†, D. confragosa†, C. cerebella†*, P. lucidus†, P. hirsutus†, A. fumigatus* | D. quercina | P. versicolor, A. glaucus |
| Isaria sp.? | | M. pinastri†, A. niger† | P. sapidus, P. versicolor, P. lucidus, P. hirsutus | Isaria |
| P. lucidus | M. lacrymans*, Isaria*, A. niger* | L. vialis†, M. pinastri*, P. sapidus*, M. lacrymans†, L. lepideus†, D. confragosa†, C. cerebella*, P. versicolor†, A. glaucus†, A. versicolor† | T. Peckii, A. niger | P. lucidus |
| P. hirsutus | | M. pinastri*, P. sapidus†, D. confragosa†, P. versicolor†, A. versicolor*, A. Sydowi*, A. glaucus* | L. vialis, T. Peckii, M. lacrymans, B. cerebella, P. lucidus | P. hirsutus |
| A. glaucus | | T. Peckii*, C. cerebella*, P. hirsutus*, A. glaucus†, A. niger†, A. fumigatus*, A. versicolor† | L. vialis, T. Peckii, P. sapidus, P. lucidus, P. hirsutus | A. fumigatus, A. Sydowi |
| A. niger | | A. versicolor*, A. Sydowi†, D. quercina*, M. lacrymans†, D. confragosa*, C. cerebella*, P. lucidus*, P. hirsutus*, A. glaucus†, A. fumigatus* | T. Peckii, P. sapidus | |
| A. fumigatus | | M. pinastri†, M. lacrymans†, A. glaucus*, A. versicolor†, A. Sydowi† | T. Peckii, Isaria, P. lucidus | D. confragosa, A. fumigatus |
| A. versicolor | | P. hirsutus*, A. fumigatus†, A. glaucus† | P. sapidus, A. Sydowi | P. versicolor, A. glaucus |
| A. Sydowi | | P. hirsutus*, A. niger†, A. fumigatus† | P. sapidus, Isaria, P. lucidus, A. versicolor | |

*=after contact; † =before contact.

of the fungi the plates were cut into small squares (about 8 mm. square), which were used as inocula. Agar plates made in a similar manner were each inoculated with three of the fungi in such a way as to have all possible combinations of each fungus. From these plates the reciprocal influence of growth was determined. The results of the plate cultures are shown in table I.

The outstanding feature of these results is the preponderance of inhibition of growth of one fungus before and after contact with another. In some cases this inhibition took place when the two colonies were still a considerable distance apart; in others only when they came into close proximity with each other. Figures 5 and 2 respectively of pl. 4 illustrate this feature. In those cases where inhibition occurred after contact the condition is shown by a straight line unless one fungus has a much more rapid growth than the other. Figures 8 and 11 illustrate this point. It often happened that one fungus on the plate grew much more rapidly than the other two, cutting off contact between them. Therefore, all possible combinations could not be recorded in the table.

There were not as many instances where one fungous colony grew over another as there were of inhibition of growth. In some cases of the former type one colony was completely covered, and the shape of the submerged colony determined that of the colony of the invading fungus, as, for example, in the case of *Pleurotus sapidus* growing over *Aspergillus glaucus* and *A. Sydowi* (see figs. 3 and 6). In these cases the growth of *Pleurotus sapidus* is greatly accelerated as soon as it reaches the colonies. In other cases the growth of the invading fungus was comparatively slow, as, for example, when *Pleurotus sapidus* invaded a colony of *Aspergillus niger* (see fig. 9). At first both of these fungi were mutually inhibited and then *Pleurotus sapidus* gradually advanced. A peculiarity of this special case is the fact that the spores of *Aspergillus niger* disappeared in the invaded section. It could not be determined whether these spores germinated or were digested.

When two colonies of the same fungus came into contact there was usually no influence of the one colony on the other; that is,

the mycelium of the two thoroughly intermixed, as fig. 12 in the plate shows for *Merulius pinastri*. An exception to this general condition is illustrated when two colonies of *Aspergillus niger* grew together. At first there was an inhibition of growth as shown by a straight line formed by the margins of the two colonies. Later, however, the two colonies generally intermixed. As table I shows, there was often an intermixing of the mycelium of two different species. This may be explained by the theory advanced by Clark[1] that many deleterious substances, which at certain concentrations retard growth, later cause great acceleration of mycelial development in the retarded cultures.

Cases of stimulation of growth when two colonies came into contact were comparatively rare, while cases of stimulation before contact occurred seldom indeed. However, examples of both these types were observed. In many cases it is hard to distinguish between true stimulation and a mere heaping up of the mycelium due to mechanical hindrance. Figures 7 and 10 of the plate show a stimulation of *Trametes Peckii* in contact with *Daedalea quercina*. At first there was a heaping up of the mycelium, and this appeared to be a great stimulation of growth. However, this may equally well be considered a mere increase in the amount of aërial mycelium due to a mechanical hindrance of the surface of the medium. A peculiar case of stimulated growth of *Daedalea confragosa* is shown in fig. 1. It is not certain whether this is caused by the presence of *Merulius lacrymans* or some other factor. However, there does not seem to be any valid reason why a stimulation by diffusion should not be expected as much as inhibition of growth by diffusion, as where *Lentinus lepideus* and *Aspergillus glaucus* are mutually inhibited before contact. In the latter case the colonies never came together. A slight stimulation of growth of *Polyporus lucidus* in the neighborhood of *Isaria* is shown in fig. 4.

It was noticed that the sporulation of certain of the *Fungi Imperfecti* was influenced by the growth of other fungi. For example, there seemed to be an increase in size and number

[1] Clark, J. F. On the toxic effect of deleterious agents on the germination and development of certain filamentous fungi. Bot. Gaz. 28: 289–327, 378–404. 1899.

of the heads of conidiospores of *Aspergillus Sydowi* when in contact with *Merulius pinastri*, and the same is true of *Aspergillus niger* in contact with *A. glaucus*.

As previously mentioned, hydrogen ion concentrations of solutions were determined after fungi had grown on them for two weeks, and the results obtained indicate that there is no definite relation between the active acidity produced by these fungi and their ability to inhibit or stimulate the growth of another. For example, in fig. 3, *Pleurotus sapidus* grew over *Aspergillus glaucus* very rapidly, but was entirely inhibited by *A. versicolor* on the solid agar medium. In the solutions *Pleurotus sapidus* produced an active acidity of $P_H$ 5.4, while *Aspergillus glaucus* and *A. versicolor* changed the active acidity to about the degree $P_H$ 6.6 and $P_H$ 6.4, respectively; also, *Trametes Peckii* grew over both *Daedalea quercina* and *Aspergillus fumigatus*, although the change in active acidity produced by *Daedalea quercina* was $P_H$ 3.0 and that produced by *Aspergillus fumigatus* was $P_H$ 6.6. Many such examples could be cited by comparing with table I the following active acidities produced by the fungi: *Lenzites vialis*, $P_H$ 5.0; *Merulius pinastri*, $P_H$ 7.0; *Daedalea quercina*, $P_H$ 3.0; *Trametes Peckii*, $P_H$ 4.2; *Pleurotus sapidus*, $P_H$ 5.4; *Merulius lacrymans*, $P_H$ 5.0; *Lentinus lepideus*, $P_H$ 5.4; *Daedalea confragosa*, $P_H$ 5.8; *Coniophora cerebella*, $P_H$ 5.4; *Polystictus versicolor*, $P_H$ 5.4; *Isaria* sp., $P_H$ 6.8; *Polyporus lucidus*, $P_H$ 5.4; *Polystictus hirsutus*, $P_H$ 5.2; *Aspergillus glaucus*, $P_H$ 6.6; *A. niger*, $P_H$ 5.8; *A. fumigatus*, $P_H$ 6.6; *A. versicolor*, $P_H$ 6.4; and *A. Sydowi*, $P_H$ 6.8. The control solution upon which no fungi had grown had an active acidity of $P_H$ 5.4. Of course, there are some instances where similar effects could be correlated with similar changes in hydrogen ion concentration; for instance, *Trametes Peckii* is similarly influenced by both *Aspergillus fumigatus* and *A. glaucus*.

The fungi were also grown on a nutrient solution containing the same ingredients as the agar previously mentioned. Since certain of the *Basidiomycetes* used do not grow well upon liquid media it was found desirable to add to the cultures sufficient quartz sand, free from all soluble substance, so that a slope of sand could be formed above the surface of the solution out into

which the solution diffused. All of the *Basidiomycetes* grew well upon these sand slopes, and the solution was easily drained from the sand at the end of the period of culture. After two weeks' growth of the fungi the hydrogen ion concentration of the solutions was determined according to the methods previously cited.[1]

In cases where there was a marked stimulation or inhibition of growth between two fungi on the plates, these fungi were then grown on similar sand slopes. After they had made considerable growth the solution was filtered off, sterilized, and prepared for inoculation with the reciprocal fungus. Controls of these solutions were kept uninoculated. The amount of growth of the second inoculation was determined by the dry weight of the fungous mat and the amount of sugar remaining in the solutions estimated. The latter was accomplished by reducing equal amounts of the solutions with equal amounts of Fehling's solution and estimating visually the amounts of copper oxide. The distinctions were so evident that quantitative determinations were unnecessary.

The dry weight of mycelium produced in each case and an estimation of the amount of sugar remaining in the solution after growth of the first and second fungus are shown in table II.

In some cases it would seem that the carbohydrate content of the nutrient solution upon which a fungus had previously grown might have been the limiting factor for growth. In others, however, this is not true; for example, when *L. vialis* follows *A. niger* there is very little growth, although the carbohydrate content was high, while in the control solution upon which no fungus had grown *L. vialis* made considerable growth and used a greater part of the sugar in the solution. This would tend to indicate that *A. niger* in its metabolism may have secreted some substance which was toxic to the growth of *L. vialis*. It is of course quite probable that such toxic substances were formed in many more instances but were destroyed in the process of autoclaving between the first and second inoculation.

This in general agrees with the conclusions reached by Fulton[2] that fungi in their growth show a more marked tendency to grow

[1] Zeller, Schmitz, and Duggar, *l. c.*

[2] Fulton, H. R. Chemotropism of fungi. Bot. Gaz. 41:81–108. 1906.

## TABLE II

RELATION OF THE AMOUNT OF GROWTH TO THE QUANTITY OF SUGAR REMAINING IN THE SOLUTION

| Fungus | Grown on solution after | Dry weight of mycelium (gms.) | Relative amounts of remaining sugars |
|---|---|---|---|
| L. vialis | A. niger | .080 | Much |
| | A. Sydowi | .062 | Trace |
| | Control | .221 | Medium |
| M. pinastri | T. Peckii | .115 | Trace |
| | A. niger | .040 | Trace |
| | Control | .119 | None |
| T. Peckii | M. lacrymans | .154 | Much |
| | P. sapidus | .335 | Much |
| | Isaria | .140 | Medium |
| | A. niger | .084 | Trace |
| | A. fumigatus | .118 | Medium |
| | A. glaucus | .126 | Medium |
| | Control | .221 | Much |
| P. sapidus | A. niger | .302 | None |
| | A. glaucus | .180 | Trace |
| | A. Sydowi | .309 | Trace |
| | Control | .353 | Much |
| M. lacrymans | T. Peckii | .178 | Trace |
| | D. confragosa | .100 | Trace |
| | Control | .230 | Much |
| L. lepideus | T. Peckii | .094 | Medium |
| | A. niger | .073 | Trace |
| | A. fumigatus | .100 | Trace |
| | Control | .111 | Much |
| D. confragosa | M. lacrymans | .290 | Trace |
| | Control | .231 | Much |
| P. versicolor | P. sapidus | .372 | Trace |
| | A. glaucus | .085 | None |
| | A. Sydowi | .088 | Trace |
| | Control | .266 | None |
| P. lucidus | Isaria | .117 | Trace |
| A. niger | P. sapidus | .270 | Medium |
| | M. lacrymans | .240 | Trace |
| | D. confragosa | .141 | Trace |
| | C. cerebella | .270 | Trace |
| | A. fumigatus | .078 | None |
| | Control | .270 | Trace |
| A. fumigatus | C. cerebella | .156 | Much |
| | Control | .158 | Trace |

out and *away from* the medium influenced by their own growth metabolism than to grow *towards* a diffusion center, whether this center contains nutritive or deleterious materials. This may also be the condition produced in stale cultures.

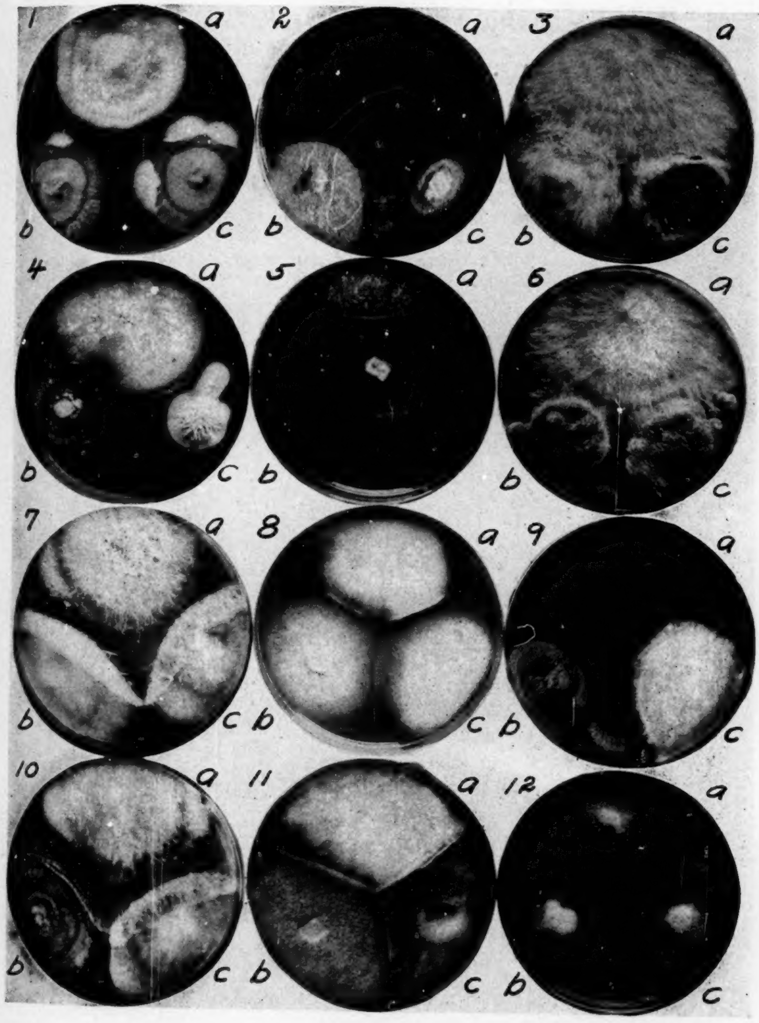
Thanks are due to the Missouri Botanical Garden for the facilities of the library and the laboratories and to Dr. B. M. Duggar for helpful co-operation.

*Graduate Laboratory, Missouri Botanical Garden.*

## Explanation of Plate

### PLATE 4

Fig. 1. *a, Merulius lacrymans; b* and *c, Daedalea confragosa.*
Fig. 2. *a, Aspergillus niger; b, A. fumigatus; c, Lentinus lepideus.*
Fig. 3. *a, Pleurotus sapidus; b, Aspergillus glaucus; c, A. versicolor.*
Fig. 4. *a, Polyporus lucidus; b, Lenzites vialis; c, Isaria* sp.
Fig. 5. *a, Aspergillus fumigatus; b, Lentinus lepideus.*
Fig. 6. *a, Pleurotus sapidus; b, Aspergillus Sydowi; c, A. glaucus.*
Fig. 7. *a, Trametes Peckii; b* and *c, Daedalea quercina.*
Fig. 8. *a, Polyporus lucidus; b, Polystictus hirsutus; c, Trametes Peckii.*
Fig. 9. *a, Aspergillus niger; b, A. fumigatus; c, Pleurotus sapidus.*
Fig. 10. *a, Trametes Peckii; b, Lenzites vialis; c, Daedalea quercina.*
Fig. 11. *a, Polyporus lucidus; b, Polystictus hirsutus; c, Merulius pinastri.*
Fig. 12. *a, Daedalea quercina; b* and *c, Merulius pinastri.*




ZELLER AND SCHMITZ—MIXED CULTURES

# STUDIES IN THE PHYSIOLOGY OF THE FUNGI

## IX. Enzyme Action in Armillaria mellea Vahl, Daedalea confragosa (Bolt.) Fr., and Polyporus lucidus (Leys.) Fr.

HENRY SCHMITZ

*Formerly Rufus J. Lackland Fellow in the Henry Shaw School of Botany of Washington University*

AND SANFORD M. ZELLER

*Formerly Visiting Fellow in the Henry Shaw School of Botany of Washington University*

Careful studies in the physiology of the wood-destroying fungi have but recently received the degree of consideration to which their economic importance entitles them. Many economic forms are as yet untouched, and it is our purpose to study some of the fundamental physiological relations existing between fungus and host. The following is the first of a series of investigations concerning especially the enzyme activities of such forms. It is recognized that *Armillaria mellea* has received considerable attention in respect to its physiological relations because of its importance as a root rot of fruit trees, but, as far as the writers are aware, there has been no physiological study of *Daedalea confragosa* or *Polyporus lucidus*, both of which must be recognized as important wood-rotting fungi.

In a recent paper by one of us[1] the literature and methods of enzyme study in the wood-destroying fungi have been sufficiently reviewed, so that in the present paper only specific references to previous literature will be made, and unless otherwise stated, the methods followed will be those previously described.

The fungi from which was obtained the fungous meal used in the present study were grown on sterile, sliced carrot in large Erlenmeyer flasks. While still in an active growing condition the fungous mats were removed and rapidly air-dried by means

[1] Zeller, S. M. Studies in the physiology of the fungi. II. Lenzites saepiaria Fries, with special reference to enzyme activity. Ann. Mo. Bot. Gard. 3: 439–512. *pl. 8–9.* 1916.

of an electric fan. When thoroughly dry the material was finely ground.

## Esterases

In the study of the esterases of these three fungi methyl acetate, ethyl acetate, ethyl butyrate, triacetin, and olive oil emulsion were used as substrates. When esterases act upon esters fatty acids are liberated, and thus the concentration of the active acidity can be used as an index of the degree of enzyme action. First, a determination was made of the hydrogen ion concentration of the substrate. A similar determination was also made of the substrate to which a certain amount of autoclaved fungous meal had been added. These two determinations did not always check, due to the introduction of certain substances with the fungous meal and perhaps also to certain buffer effects. The latter determination was taken as the control in each case and compared with a third determination made of the substrate to which a similar amount of fungous meal had been added and incubated twenty-one days.

It was found that there was no apparent esterase activity of any of the fungi on any of the substrates except methyl acetate upon which a slight esterase activity was shown in the case of *Daedalea confragosa* and *Polyporus lucidus*. These results are similar to those found for *Lenzites saepiaria*.[1]

## Carbohydrases

The action of carbohydrases was determined upon maltose, lactose, sucrose, raffinose, potato starch, inulin, cellulose from various sources, and hemicellulose. The amount of sugars which reduce Fehling's solution in the enzyme cultures after incubation was taken as the index of enzyme activity. Since this study is merely to indicate the relative activity between the different fungi on the different substrates the results are given as the number of cc. of N/20 potassium permanganate required to oxidize the dissolved copper oxide. The results in the following table are the averages of duplicate enzyme cultures after the Fehling's control had been deducted.

[1] Zeller, S. M., *l. c.*

TABLE I

SHOWING THE ACTION OF CARBOHYDRASES ON POLYPORUS LUCIDUS, ARMILLARIA MELLEA, AND DAEDALEA CONFRAGOSA

| Incubation period | Substrate | P. lucidus | | | A. mellea | | D. confragosa | |
|---|---|---|---|---|---|---|---|---|
| | | With fungous meal | With fungous meal autoclaved | Without fungous meal | With fungous meal | With fungous meal autoclaved | With fungous meal | With fungous meal autoclaved |
| | | Number of cc. of $\frac{N}{20}$ $KMnO_4$ | | | | | | |
| 14 days | Maltose | 36.1 | 16.3 | 13.1 | 36.2 | 16.3 | 23.5 | 14.7 |
| 24 days | Lactose | 31.7 | 23.0 | 18.8 | 36.9 | 26.0 | 24.9 | 19.3 |
| 6 hours | Sucrose | 27.5 | 1.6 | 1.0 | 31.3 | 2.3 | 11.1 | 0.3 |
| 2 days | Raffinose | 32.5 | 5.2 | 0.8 | 12.5 | 2.6 | 12.5 | 0.6 |
| 6 hours | Potato starch | 19.2 | 6.1 | 1.4 | 25.3 | 2.6 | 7.7 | 1.5 |
| 2 days | Inulin | 20.3 | 4.9 | 0.8 | 15.4 | 2.4 | 7.8 | 0.9 |
| 28 days | Ash cellulose | 5.6 | 0.7 | 0.3 | 4.1 | 0.5 | 4.3 | 1.8 |
| 28 days | Fir cellulose | 5.8 | 0.3 | 0.2 | 3.3 | 0.4 | 4.7 | 0.4 |
| 28 days | Oak cellulose | 3.8 | 0.2 | 0.1 | 3.6 | 0.1 | 3.1 | 1.3 |
| 28 days | Hemi-cellulose | 4.4 | 0.3 | 0.1 | 2.9 | 0.1 | 3.2 | 1.8 |

In general the carbohydrate activity is greater in *Polyporus lucidus* and *Armillaria mellea* than in *Daedalea confragosa*, with the possible exception of raffinase where the activity in *Daedalea confragosa* approximates that of *Armillaria mellea*. The striking feature of the results is the evident presence of lactase in the three fungi. This is the first record of the presence of lactase in the higher fungi.

In the study of cellulase pure cellulose was prepared from Douglas fir, ash, and red oak, according to the method frequently reported from this laboratory. Suspensions of these in doubly

distilled water were used as substrates. After the enzyme culture had been incubated for 28 days there was a marked increase in the amounts of reducing sugars produced by all the fungi on all of the substrates.

For a hemicellulose substrate cleaned autoclaved endosperms from date seeds were used. These were shaved into very thin slices and placed in distilled water with the fungous meal. Hydrolysis quite comparable to that produced in the cellulose experiments resulted in each case as is indicated in table I.

## EMULSIN

The presence or absence of emulsin was determined by the effect of the fungous meal upon amygdalin, which upon hydrolysis produces glucose, benzaldehyde, and hydrocyanic acid. After incubation of seven days the amount of glucose present in the cultures was determined as in the cases where carbohydrates were used as substrates, and the results are tabulated in the following table:

TABLE II

SHOWING THE ACTION OF EMULSIN ON POLYPORUS LUCIDUS, ARMILLARIA MELLEA, AND DAEDALEA CONFRAGOSA

| Enzyme culture | P. lucidus | A. mellea | D. confragosa |
|---|---|---|---|
| | Number of ce. of $\frac{N}{20}$ $KMnO_4$ | | |
| 1% amygdalin+ fungous meal | 26.0 | 12.5 | 18.1 |
| 1% amygdalin+ fungous meal (autoclaved) | 4.8 | 0.6 | 0.1 |
| 1% amygdalin | 0.6 | 0.6 | 0.6 |

In all cases where the above sugar tests showed evidence of the breaking down of amygdalin the odor of benzaldehyde was easily recognized. There was evidence of emulsin in all three of the fungi used.

## TANNASE

In order to determine the tannase activity the gallic acid, which is a product of hydrolysis of tannic acid, was titrated with

standard iodine solution. The results show the presence of tannase in *Polyporus lucidus* and *Daedalea confragosa* but none was detected in *Armillaria mellea.* This fact seems peculiar, since the rhizomorphs of *Armillaria mellea* are usually found next to the inner bark of the woody tissues where the tannin is usually present in the greatest amount for the specific host. The fact that the fungi from which the fungous meal was made were grown on carrot may have had some influence on the production of tannase in this particular instance.

### Amidase and Urease

The presence of the enzymes which split amino acids and urea into ammonia and hydroxy acids was demonstrated by using such substrates as asparagin, acetamid, and urea. The usual Folin method of determining the presence of ammonia is such a time-consuming procedure that a new method was devised involving the indicator method of determining the hydrogen ion concentration of solutions. In brief, the method employed is as follows:

The substrate and fungous meal in the desired proportions were placed in wash bottles, the inlets and outlets of which were sealed with rubber tubes and clamps in order to retain any ammonia which might have been given off during the period of incubation. After a period of incubation of seven days the ammonia was drawn directly through another small wash bottle by means of a Richards pump. The small wash bottle contained 10 cc. of doubly distilled water to which was added 6 drops of brom thymol blue made up in the proportions suggested by Clark and Lubs.[1] This doubly distilled water had a hydrogen ion concentration of $P_H$ 5.6, at which concentration the indicator was yellowish brown. Due to the hydrogen ion concentration decreasing as the ammonia is drawn through, the color changes from green to blue. The length of time taken to change from $P_H$ 5.6 to $P_H$ 7.0 would, of course, depend upon the amount of ammonia present, and this was thus taken as a criterion of the

[1] Clark, W. M., and Lubs, H. A. The colorimetric determination of hydrogen ion concentration and its applications in bacteriology. Jour. Bact. 2: 1–34, 109–136, 191, 236. 1917.

relative rate of ammonia production in the various enzyme cultures. In no case was the gas drawn through the wash bottle for a period longer than three minutes. At the end of this period the actual hydrogen ion concentration of the distilled water was determined. Sometimes the change was so rapid that it was not necessary to run the experiment for three minutes. In such instances other indicators having a wider alkaline range were substituted for brom thymol blue. In the urea control there was a change from $P_H$ 5.6 to $P_H$ 6.0, and thus, for urea, changes not going beyond $P_H$ 6.0 were considered as negative. The results are tabulated in table III.

Urease was demonstrated for the three fungi. It was most pronounced in *Daedalea confragosa* and least in *Polyporus lucidus*. Only *Armillaria mellea* showed slight amidase action when acetamid was used as a substrate. There was no amidase action when asparagin was used as a substrate.

Due to the fact that traces of alkalis or acids cause considerable shifting of the hydrogen ion concentration in such an unbuffered solution as doubly distilled water it is believed that this method can be used to determine the presence of minute traces of ammonia which would be undetectable by the methods usually employed, and the determination is much more rapid. In the present paper only relative determinations were necessary but there is no valid reason why quantitative determinations could not be made by this method.

## Proteases

Tryptic and ereptic fermentation was studied by the use of albumin, peptone, casein, legumin, and fibrin in enzyme cultures having a neutral, acid, and alkaline reaction. When fibrin was used as a substrate positive results were obtained to show the presence of both trypsin and erepsin in all three of the fungi. These results were most pronounced in the cultures having an acid reaction and least in those with an alkaline reaction. In the case of the plant protein, legumin, there was very slight indication of the presence of tryptic and ereptic fermentation only in *Polyporus lucidus* and only when the substrate was acid in reaction. In *Polyporus lucidus* there was indication of the

presence of trypsin when albumin of acid reaction was used. This was not true of *Armillaria mellea* or *Daedalea confragosa*. In no case was there a splitting of peptone or casein.

TABLE III

UREASE AND AMIDASE ACTIVITY IN ARMILLARIA MELLEA, DAEDALEA CONFRAGOSA, AND POLYPORUS LUCIDUS

| | Enzyme culture | No. | Change in H ion concentration. $P_H$ values | | |
|---|---|---|---|---|---|
| | | | Urea | Acetamid | Asparagin |
| P. lucidus | Substrate + fungous meal | 1 | 5.6–6.8 3 min. | 5.6–5.6 3 min. | 5.6–5.6 3 min. |
| | | 2 | 5.6–6.6 3 min. | 5.6–5.6 3 min. | 5.6–5.6 3 min. |
| | Substrate + fungous meal (autoclaved) | 3 | 5.6–6.0 3 min. | 5.6–5.6 3 min. | 5.6–5.6 3 min. |
| | | 4 | 5.6–5.8 3 min. | 5.6–5.6 3 min. | 5.6–5.6 3 min. |
| A. mellea | Substrate + fungous meal | 1 | 5.6–7.2 30 sec. | 5.6–6.0 3 min. | 5.6–5.6 3 min. |
| | | 2 | 5.6–7.2 25 sec. | 5.6–6.0 3 min. | 5.6–5.6 3 min. |
| | Substrate + fungous meal (autoclaved) | 3 | 5.6–6.0 3 min. | 5.6–5.6 3 min. | 5.6–5.6 3 min. |
| | | 4 | 5.6–6.0 3 min. | 5.6–5.6 3 min. | 5.6–5.6 3 min. |
| D. confragosa | Substrate + fungous meal | 1 | 5.6–7.2 1 sec. | 5.6–5.6 3 min. | 5.6–5.6 3 min. |
| | | 2 | 5.6–8.8 3 sec. | 5.6–5.6 3 min. | 5.6–5.6 3 min. |
| | Substrate + fungous meal (autoclaved) | 3 | 5.6–6.0 3 min. | 5.6–5.6 3 min. | 5.6–5.6 3 min. |
| | | 4 | 5.6–6.0 3 min. | 5.6–5.6 3 min. | 5.6–5.6 3 min. |
| Control | Substrate alone | 1 | 5.6–6.0 3 min. | 5.6–5.6 3 min. | 5.6–5.6 3 min. |
| | | 2 | 5.6–6.0 3 min. | 5.6–5.6 3 min. | 5.6–5.6 3 min. |

SUMMARY

In *Polyporus lucidus* the presence of the following enzymes is demonstrated; esterase, maltase, lactase, sucrase, raffinase, diastase, inulase, cellulase, hemicellulase, emulsin, tannase, urease, and trypsin and erepsin when fibrin is used as a substrate.

In *Armillaria mellea* the presence of the following enzymes is demonstrated: maltase, lactase, sucrase, raffinase, diastase, inulase, cellulase, hemicellulase, emulsin, urease, amidase, and trypsin and erepsin when fibrin is used as a substrate.

In *Daedalea confragosa* the following enzymes are present: esterase, maltase, lactase, sucrase, raffinase, diastase, inulase, cellulase, hemicellulase, emulsin, tannase, urease, and trypsin and erepsin when fibrin is used as a substrate.

A new method for the determination of ammonia liberated by amidase is described. This method involves the application of the indicator method for hydrogen ion concentration determination.

Thanks are due to the Missouri Botanical Garden for the privileges of the library and laboratories and to Dr. B. M. Duggar for advice and helpful criticisms.

*Graduate Laboratory, Missouri Botanical Garden.*

# STUDIES IN THE PHYSIOLOGY OF THE FUNGI

## X. Germination of the Spores of Certain Fungi in Relation to Hydrogen Ion Concentration[1]

ROBERT W. WEBB

*Rufus J. Lackland Fellow in the Henry Shaw School of Botany of Washington University*

### Introduction

The hydrogen ion concentration of culture media or solutions has come to be regarded in recent years as one of the most important factors influencing physiological phenomena. A voluminous literature is found dealing with the toxic properties of H and OH ions in a general way, but the earlier investigators, like many of the later ones, were handicapped by lack of methods, or experience with methods, for the direct determination of hydrogen ion concentration. With such limitation in technique, conductivity data have frequently been employed in the interpretations made. This method is, however, inapplicable when other solutes are introduced, and the presence of strong buffers, whether inorganic or organic, would render most difficult any computation of active acidity or alkalinity.

Some of the questions which are unanswered are: What is the effect of hydrogen ion concentration upon the rate of germination of the spores of certain fungi, or, what is the range within which the most favorable germination occurs? Such questions suggested the desirability of conducting the investigation reported in this paper, and the scarcity of definite literature dealing with this particular phase has been one of the greatest incentives to the pursuance of the problem.

### Review of Literature

Clark ('99) seems to have been one of the pioneer workers on the toxicity of acids, alkalis, oxidizing agents, and salts of the heavier metals, towards the growth of certain fungi. Using the

[1] An investigation carried out at the Missouri Botanical Garden in the Graduate Laboratory of the Henry Shaw School of Botany of Washington University, and submitted as a thesis in partial fulfillment of the requirements for the degree of master of arts in the Henry Shaw School of Botany of Washington University.

hanging-drop method, he determined, in a nutrient medium, approximately the relative and absolute toxic properties of many deleterious agents as shown by their influence on spore germination, mycelial development, and fructification. The medium used throughout this study was an infusion of sugar beet, as experiments have shown this to be the most suitable and satisfactory medium for all the forms. The toxicity of the various acids and alkalis towards moulds is shown in detail, and interest centers upon (1) the average inhibiting concentration for germination and development, and (2) the average killing concentration, the organisms employed being *Aspergillus flavus*, *Sterigmatocystis nigra*, *Oedocephalum albidum*, *Penicillium glaucum*, and *Botrytis vulgaris*.

It was a general rule with all acids and alkalis that when the concentrations were not sufficient to cause distinct injury, stimulation of growth followed the slight retardation, such cultures taking on new vigor and surpassing the controls. Spores of *Botrytis* were most easily killed, while those of *Penicillium* offered the greatest resistance. Comparing the results with conductivity data, Clark concludes that the OH ion is more toxic towards fungi than the H ion. It is to be remembered, however, that the hydrogen ion concentration of none of his solutions was known. Nevertheless, this is of special interest when taken in conjunction with data which I shall present later.

Using distilled water as a medium, Stevens ('98) studied the effect of salts, bases, and acids upon the germination of the following fungous spores: *Botrytis vulgaris*, *Macrosporium* sp., *Penicillium crustaceum*, *Gloeosporium musarum*, and *Uromyces caryophyllinus*. As might be expected, germination of most of these species is not perfect in distilled water or else there is considerable variability in the results. Abnormal and distorted mycelium appear more frequently in the acid solution than in ordinary nutrient media. With *Macrosporium* and *Penicillium* neither HCl nor $H_2SO_4$ prevented growth, and the behavior of *Uromyces* towards these acids was quite variable. Further work with alkalis tends to indicate that KOH, NaOH, and $NH_4OH$ have a low toxic value. *Penicillium* generally offered the greatest resistance to the different agents. From the data obtained,

the author concludes that various fungi exhibit varying degrees of resistance to poisons, and that the limits of resistance may vary in the species. Even though little data were at hand, Stevens concluded that the spores of fungi, when compared to the roots of seedlings, were less susceptible to toxic action.

Duggar ('01) made an extensive study of spore germination, including certain chemical as well as physical stimuli. Using distilled water as the medium, he found that organic acids stimulated germination but the percentage of germination was not great. The stimulus of N/100 or less of acetic acid to *Aspergillus flavus* and *Sterigmatocystis nigra* was very noticeable. Oxalic acid was more pronouncedly stimulating with *Sterigmatocystis*, N/100 producing maximum germination, whereas this concentration totally inhibited germination of *Aspergillus*. In this work, the considerable extent of individual variation was emphasized.

Ferguson ('02), using an artificial digestive fluid containing solutions of pepsin in distilled water combined with different amounts of HCl also in distilled water, studied the germination of spores of *Agaricus campestris*, *Coprinus comatus*, and *Calvatia cyathiforme*, but germination was so erratic that she was unable to draw any definite conclusions.

Brooks ('06) studied the effect of temperature on the toxic properties of $CuSO_4$, $HNO_3$, and $H_2SO_4$, as shown by the effect of these substances on the germination and growth of certain fungi. In all the experiments, beet decoction was used as the nutrient medium, the stock infusion containing 600 gms. of beets per 1000 cc. of water. At the time of using, the decoction was diluted by the addition of the toxic substance and water to one-half of its former nutritive value. Usually, above the provisional optimum, the deleterious action of the toxic agents increased very rapidly with rise in temperature. Spores inhibited by cold were not greatly injured when exposed to harmful agents. In all instances, however, the injurious effects were least at the optimum temperature for the fungus; however, the effects of the three chemicals were very different.

Ayers ('16), making cultures of streptococci in a broth containing 1 per cent cerevisine, 1 per cent peptone, 1 per cent test

substance (glucose, lactose, etc.), and distilled water, obtained data indicating that streptococci reach more or less definite hydrogen ion concentration and that there are two limiting zones, $P_H$ 4.6–4.8 and $P_H$ 5.5–6.0. A very large percentage of the streptococci from cases of human infection reached only the lower limit of hydrogen ion concentration, a fact that is very striking.

Morgan and Gruzit ('16) found that soil solutions adjusted to various reactions by N/100 mineral acid and N/100 alkali, when mixed with sterile quartz, showed variations in the type and number of bacteria. A solution with N/1000 alkali gave the best growth while N/1200 acid exerted marked toxicity. In alkaline solutions the number of bacteria increased up to the point of faint alkalinity and then decreased after passing this point; whereas in acid solutions the number of bacteria increased with decrease in acidity.

Zeller ('16) found that the reaction of the medium was a most important factor influencing the growth and metabolism of *Lenzites saepiaria*. A medium of Thaxter's glucose-potato-hard agar possessing the faintest alkalinity failed to produce the slightest growth, but, on being readjusted to slight acidity, it gave good growth. Spaulding ('11) found that the same organism was unusually sensitive to alkaline media, and obtained luxuriant growth with one-fourth of 1 per cent $H_2SO_4$. Other investigators have published similar results.

Salter ('16) found that the reproduction of legume bacteria in Ashby's mannite solution and in a soil solution was greatly influenced by the reaction of the medium. A neutral or slightly acid reaction in mannite solution, the means of determination of which are not stated, proved to be the most favorable for the production of the red clover organism. Inhibition of growth was evident in slightly alkaline solutions, and no growth was found in the presence of 1 per cent normal alkali. *Bacillus radicicola* from alfalfa, on the other hand, exhibited great sensitiveness towards acidity, retardation of growth being noticed with .5 per cent normal $H_2SO_4$. The organism grew best in faintly alkaline or neutral mannite solution.

Clark and Lubs ('17) grew *Aspergillus niger* on a medium con-

sisting of 1 gm. $KH_2PO_4$, 3 gms. $NaNO_3$, .5 gm. $MgSO_4$, 100 gms. sucrose in 1 liter water, and on the seventh day found the hydrogen ion concentration to be $2\times10^{-2}$. They comment upon Waterman's estimate that the critical limit for *Penicillium glaucum* is about $1\times10^{-5}$ N. H. and for *Aspergillus niger* about $4.5\times10^{-5}$ N. H. The reviewers think that the only explanation for such discordant results must lie in a confusion in the method of expressing hydrogen ion concentration.

Fred and Loomis ('17) found that a mannitol solution with a neutral reaction gave the highest count of *B. radicicola* from alfalfa. The addition of small amounts of alkali did not appreciably alter the number of bacteria; however, acid in equivalent amounts either retarded or inhibited growth. From the curve of hydrogen ion concentration, they are inclined to think that the apparent resistance of the legume bacteria to alkali is due to the slight concentration of hydroxyl ions in the mannitol solution. This work confirms that of Salter.

Gruzit ('17) studied the effect of acids and alkalis on soil bacteria in soil solution, and found that soil bacteria were extremely sensitive to an acid reaction. $H_2SO_4$ at a concentration of N/1200 destroyed about 99.6 per cent of the bacteria; N/1400 killed about 93.0 per cent of the organisms; and N/2840 prevented the growth of about 43.0 per cent. On the other hand, N/1000 alkali gave the maximum number of bacteria.

Taylor ('17) determined the concentrations of a few organic and inorganic acids necessary to check the growth of various organisms. He obtained data which led him to conclude that there is a great variation or specificity in their activity toward different organisms.

Wolf and Harris ('17) observed that the acidity of the medium may either delay or entirely stop the growth of *B. perfringens* and *B. sporogenes*, the critical concentration of the former being $P_H$ 4.82 and the latter, $P_H$ 4.94. All the acids tested gave very similar effects and showed practically no specific qualities.

Wright ('17) studied the importance of uniform culture media, and obtained data which clearly emphasize the many discrepancies that exist when the culture medium is adjusted by means of phenolphthalein titration. He found that the hydrogen ion

concentration of the culture medium and the resistance of organisms to the action of disinfectants afford a definite relation, the greatest resistance being obtained with a culture medium having a hydrogen ion concentration $P_H$ 6.0–7.0.

Fred and Davenport ('18) found that the growth of the nitrogen-assimilating bacteria in culture solutions of different reactions was related to the hydrogen ion concentration of the medium. Of the legume bacteria, the organisms of alfalfa were the most sensitive to hydrogen ion concentration, the limit of growth on the acid side being between $P_H$ 5.4 and 5.6; while, on the contrary, the organisms of lupine were the most resistant, the limit of growth on the acid side being $P_H$ 4.6. Sodium hydroxide did not cause any noticeable toxicity towards the legume bacteria until added in greater quantities than N/125 and appeared to have only one-tenth the toxic properties of $H_2SO_4$ towards these organisms. The authors cite Beijerinck as having secured optimum growth of *Rhizobium leguminosarum* in N/166.6 acid, but explain the disagreement of results on the ground of employing different culture media. *Azotobacter* proved to be very sensitive to slight changes of reaction and was able to grow only within the narrow limits, $P_H$ 6.5–8.6.

Meacham ('18) determined the hydrogen ion concentration of synthetic and malt-extract media necessary to inhibit the growth of *Lenzites saepiaria*, *Fomes rosens*, *Coniophora cerebella*, and *Merulius lacrymans*. Growth is not inhibited until a very high hydrogen ion concentration is reached, and, while the different fungi show considerable fluctuations, the organisms respond in much the same way. In general, growth proceeds in a straight line until about $P_H$ 2.6; decreases almost abruptly at $P_H$ 2.6, the range $P_H$ 2.6–1.9 being termed the "critical range"; from $P_H$ 1.9, the decline is more gradual and the limiting $P_H$ value appears to be about 1.7. Prior to the sudden decrease at $P_H$ 2.6, there frequently occurs a maximum of growth, usually about $P_H$ 3.0.

Krönig and Paul ('97) found a solution of $HNO_3$ to be distinctly more toxic to anthrax spores than the same concentration of HCl. Their results with acetic acid were similar to those obtained by Clark ('99), but the results with alkalis were not consistent with Clark's.

## Methods

The methods employed in this investigation, as described by Clark ('99) and Duggar ('01), are substantially those used by others in this laboratory.

*Organisms.*—The fungi used were *Aspergillus niger*, *Penicillium cyclopium*, *Fusarium* sp., *Botrytis cinerea*, and *Lenzites saepiaria*. An attempt was made to use *Colletotrichum lindemuthianum*, but, owing to the failure to obtain germination in the control culture solutions, this organism was discarded. In the test-tube cultures from which the spores were obtained, the fungi were grown on potato agar made according to Duggar, Severy, and Schmitz ('17); i. e., 230 gms. of potato were cut into small pieces, autoclaved in 1 litre of water for 1 hr. at 15 lbs. pressure, filtered while hot, 15 gms. of agar added, the mixture then autoclaved for 15 minutes at 15 lbs., correction made for loss of water, and finally tubed, sterilized, and slanted. The cultures were allowed to grow at room temperature, and the spores were always taken from cultures that were from 10 to 15 days old.

*Culture solutions.*—The composition of the culture solutions was based primarily on Clark and Lubs's ('17) titration curve of ortho-phosphoric acid. Stock solutions of M/5 mannite in M/10 $H_3PO_4$ and M/5 mannite in N/5 NaOH were made. Into sterile Pyrex flasks, 100 cc. of the M/5 mannite-M/10 $H_3PO_4$ solution were placed, and increasing proportions of M/5 mannite-N/5 NaOH were added. The flasks were plugged with cotton and sterilized at 15 lbs. pressure for 15 minutes, after which the hydrogen ion concentrations were determined by the colorimetric method as outlined by Clark and Lubs ('17). The procedure was as follows:

To a test-tube containing a 10-cc. portion of the culture fluid the proper indicator was added, and the color developed compared with the colors obtained upon the addition of the same indicator to tubes containing equal quantities of standard buffer solutions. All solutions were made from the best chemicals, purified according to Clark and Lubs ('17), and made up with doubly distilled water. A series of solutions was thus obtained ranging in hydrogen ion concentration from $P_H$ 2.8 to 10.0+. In nearly every case the determined value was identical with

the calculated value, the greatest divergence being .2. In the alkaline range the concentration of the OH ions in the last solution was beyond the range of the indicator, so it has been designated as 10.0+. The ten solutions, termed a series, are as follows: $P_H$ 2.8, 3.1, 4.4, 5.0, 6.2, 7.0, 7.4, 8.8, 9.6, and 10.0+.

Small portions of each of the solutions were transferred to sterile test-tubes permanently labeled and fitted with rubber stoppers, through each of which passed a glass rod drawn to a blunt point. All transfers of a solution were made with its particular rod, thus avoiding all chances of mixing solutions. Fresh solutions were placed in the tubes from time to time, and verifications of hydrogen ion concentration frequently made.

*Method of culture.*—The method of culture was based primarily on the hanging-drop or Van Tieghem cell. The glass cylinders employed were perfectly ground at each end and measured 18.0 mm. in diameter and 9.0 mm. in height, possessing therefore a volume of 2.3 cc. The cylinders were cemented to the slide by means of wax; the tops of the cylinders were then coated with a thin ring of vaseline, and the cells completed by sealing cover glasses to the tops of the cylinders. A small nick was made in each ring of vaseline, prior to sealing, so that equilibrium of air pressure might exist when the cultures were placed in the incubator. About 15 minutes later, the cultures were examined and the cover glasses slightly pressed to the cylinders in order to insure a perfect sealing. Two cells were placed on each slide and labeled by gumming numbered and lettered labels to the center of the slip.

Four or five drops of the same solution as that to be used in the culture were placed in the bottom of the cell, the object being to establish a complete equilibrium of vapor pressures in the cells and to prevent changes in the concentration of the solution tested, as shown by Clark ('99). A few drops of the same solution were also placed on a sterile slide, and spores transferred from a pure culture to the slide by means of a sterile platinum needle. A solution of spores was thus made and thoroughly stirred in order to prevent the spores from adhering in bunches. A drop of the spore solution was transferred from the slide to the cover glass by means of a clean,

sterile, glass rod drawn to a small point. The cover glass bearing the culture was then inverted on the cell and gently pressed until completely closed with the exception of the minute opening previously described. All cultures were made up at room temperature, and, when a set of cultures was completed, all were placed in an incubator and kept at a constant temperature. Cultures of each organism were incubated at 22° C., 27° C., and 31° C., respectively, with the exception of *Lenzites saepiaria.*

*Care of cells, etc.*—The glass rings and slide composing the cells were never used a second time without being taken apart and thoroughly cleaned by boiling in alkali, soaking in cleansing mixture, and repeatedly rinsed in distilled water. They were then sterilized in an oven at 150° C. for 1 hour and protected from dust until needed. The cover glasses were treated similarly except that they were boiled longer in order to remove all traces of vaseline, and finally dipped in alcohol and wiped dry. All slides, rods, etc., were placed in water after having been used, and given the same general treatment before setting up another series.

*Examination of cultures and data.*—Cultures were made up, as a rule, in the afternoon and examined at different intervals, depending on the length of time required for the spores of a particular organism to germinate, as determined by a preliminary experiment. Spore counts were made from five fields of the hanging drop, and the average percentage of germination recorded. Where possible three different readings were made with each set of cultures, but often the mycelial growth was so luxuriant that only two were possible. All of the experiments were run in duplicate, and the germination data reported in this paper represent the percentage averages of the two cultures. Although slight fluctuations occurred throughout the work, the result from any cell agreed very closely with that of the duplicate.

*Curves.*—The curves are developed from the percentage averages, as indicated above, each curve representing the final reading of the germination quantities of a certain organism at a particular temperature. The percentages of spore germination are plotted as ordinates and the hydrogen ion concentration of

the solutions as abscissae. Curves corresponding to each of the temperatures of incubation are found in each figure, the solid line representing 22° C., the dotted line, 27° C., and the broken line, 31° C.

## EXPERIMENTAL DATA

In examining the experimental results, it must be borne in mind that perfect germination is not to be expected with these fungi in a solution containing mannite as the sole nutrient. In fact, dextrose would perhaps have yielded higher germination percentages, but it is not certain that it would remain stable with the treatment given. Moreover, in this preliminary work it was not desired to employ a full nutrient solution on account of greater difficulties of $P_H$ adjustment.

The influence of hydrogen ion concentration upon the germination of the spores of *Aspergillus niger* may be seen by referring to table I.

TABLE I

ASPERGILLUS NIGER. AVERAGE PERCENTAGES OF SPORE GERMINATION IN M/5 MANNITE AT DIFFERENT TEMPERATURES AND AT VARIOUS HYDROGEN ION CONCENTRATIONS

| Temp. | Hrs. | Hydrogen ion concentration, $P_H$ | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 2.8 | 3.1 | 4.4 | 5.0 | 6.2 | 7.0 | 7.4 | 8.8 | 9.6 |
| 22° C. | 16 | 33.1 | 64.2 | 33.4 | 19.8 | 13.7 | 9.0 | 2.0 | 0.0 | 0.0 |
| | 26 | 63.5 | 83.1 | 55.0 | 42.1 | 18.1 | 13.4 | 14.0 | 8.2 | 0.0 |
| | 38 | 66.8 | 87.5 | 55.6 | 42.9 | 20.2 | 15.5 | 15.8 | 8.6 | 0.0 |
| 27° C. | 16 | 45.3 | 69.4 | 25.9 | 20.0 | 5.5 | 15.1 | 0.0 | 0.0 | 0.0 |
| | 26 | 53.0 | 71.8 | 31.0 | 23.7 | 7.1 | 20.1 | 0.0 | 0.0 | 0.0 |
| 31° C. | 16 | 22.6 | 32.6 | 46.6 | 44.0 | 24.4 | 18.6 | 0.0 | 0.0 | 0.0 |
| | 26 | 28.1 | 50.4 | 47.7 | 45.4 | 32.0 | 22.6 | 3.0 | 0.0 | 0.0 |

Incubated at 22° C., the data show that maximum germination is obtained in the culture having a hydrogen ion concentration of $P_H$ 3.1. With further increase in hydrogen ion concentration, there is a marked inhibition of germination, the percentage decreasing from 64.2 at $P_H$ 3.1 to 33.1 at $P_H$ 2.8; while, with decrease in hydrogen ion concentration from $P_H$ 3.1, there is a

general decrease in percentage of germination. Only a comparatively small amount of germination is obtained in the culture testing $P_H$ 7.4 and no germination whatever is evident at $P_H$ 8.8. The limiting concentration of hydrogen ions lies between $P_H$ 7.4 and 8.8 on the alkaline side, and below $P_H$ 2.8 on the acid side.

Examination of the same series after an incubation of 26 and 38 hours, respectively, shows that germination has increased in all the cultures and that there have been several slight changes in the curve of germination. A slight maximum is noticed on the alkaline side at $P_H$ 7.4, and a relatively low percentage of germination is obtained at $P_H$ 8.8, which at 16 hours was the limiting concentration.

A series incubated at 27° C. for 16 hours gives a general curve of germination very similar to that incubated at 22° C. with the exception of several slight shifts. A maximum of germination is obtained at $P_H$ 3.1, as before, but the limiting concentration on the alkaline side shifts toward neutrality; thus even at $P_H$ 7.4 there is total inhibition of germination, as compared with $P_H$ 9.6 in the first series. With decrease in hydrogen ion concentration from $P_H$ 3.1 there is a general decrease in percentage of germination to culture $P_H$ 6.2 where a minimum is reached, followed by a relatively small rise at $P_H$ 7.0. Upon incubating the series for 10 additional hours, the same relations of germination are obtained. On account of the luxuriant mycelial growth, only two readings were possible with this series.

Incubation at 31° C. gives slightly different results from those incubated at 27° C. At the time of the first reading the maximum count occurs at $P_H$ 4.4, and there is no evidence of stimulation of germination in the neutral or slightly alkaline cultures. On the other hand, germination decreases with decrease in $\overset{+}{H}$ ion concentration from $P_H$ 3.1 to 7.0, the limiting concentration proving to be $P_H$ 7.4. In the final reading maximum germination on the acid side shifts to $P_H$ 3.1, thus making the maximum germination at each temperature occur at $P_H$ 3.1; and the remaining figures confirm the results of the previous examination.

These relations are shown graphically in the curves of fig. 1.

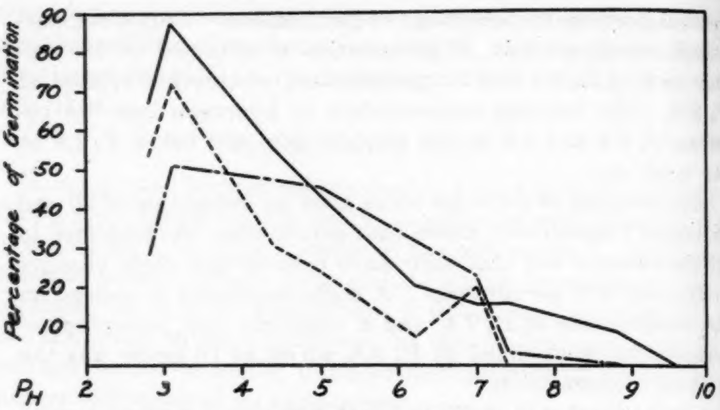



Fig. 1. *Aspergillus niger.* Graphic representation of the relation of germination to H ion concentration.

The data obtained with spores of *Penicillium cyclopium* as given in table II, are somewhat similar to those with *Aspergillus niger*.

TABLE II

PENICILLIUM CYCLOPIUM. AVERAGE PERCENTAGES OF SPORE GERMINATION IN M/5 MANNITE AT DIFFERENT TEMPERATURES AND AT VARIOUS HYDROGEN ION CONCENTRATIONS

| Temp. | Hrs. | Hydrogen ion concentration, $P_H$ | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 2.8 | 3.1 | 4.4 | 5.0 | 6.2 | 7.0 | 7.4 | 8.8 | 9.6 | 10.0+ |
| 22° C. | 18 | 4.1 | 13.5 | 16.8 | 14.0 | 7.1 | 6.6 | 10.5 | 4.4 | 2.1 | 0.0 |
| | 27 | 10.3 | 21.1 | 30.7 | 16.6 | 10.9 | 8.1 | 28.7 | 10.5 | 2.7 | 0.0 |
| | 37 | 11.4 | 22.7 | 32.3 | 23.6 | 11.9 | 8.6 | 31.9 | 13.0 | 3.1 | 1.7 |
| 27° C. | 18 | 32.2 | 35.5 | 22.9 | 20.7 | 10.5 | 12.3 | 7.0 | 0.0 | 0.0 | 0.0 |
| | 27 | 58.6 | 65.6 | 52.2 | 32.5 | 18.1 | 28.7 | 12.1 | 0.0 | 0.0 | 0.0 |
| | 37 | 59.8 | 66.6 | 55.4 | 35.1 | 22.0 | 33.2 | 18.7 | 0.0 | 0.0 | 0.0 |
| 31° C. | 18 | 15.6 | 44.2 | 29.3 | 13.4 | 9.7 | 5.6 | 9.6 | 0.0 | 0.0 | 0.0 |
| | 27 | 30.0 | 57.8 | 39.3 | 25.2 | 19.5 | 6.8 | 12.1 | 5.0 | 0.0 | 0.0 |

When incubated at 22° C. for 18 hours, maximum germination is obtained in the culture with a hydrogen ion concentration of $P_H$ 4.4, and percentage germination decreases with decrease in hydrogen ion concentration to the culture testing $P_H$ 7.0 where

minimum germination is obtained. At $P_H$ 7.4 there is a slight increase followed by a gradual decline to the culture possessing an exponent 9.6, the limiting concentration appearing to be beyond 10.0+. With increase of hydrogen ion concentration above $P_H$ 4.4, there is a decrease in percentage of germination, but the limiting concentration evidently lies above the concentration $P_H$ 2.8. Readings at incubation periods of 27 and 37 hours, respectively, give very similar results to those of the first reading, the only difference being that relatively slight germination is obtained in the culture with the value $P_H$ 10.0+, which was formerly the limiting concentration. In no other case did *Penicillium* germinate at this relatively extreme alkalinity, and, inasmuch as these spores frequently collected in bunches, it is thought that the apparently erratic germination might be due to this fact.

At 27° C. maximum germination is obtained in the culture where the exponent is $P_H$ 3.1, and the curve proceeds in the same general direction as before with the exception that minimum germination is obtained at $P_H$ 6.2, as compared with $P_H$ 7.0 in the former case. A slight rise is evident at $P_H$ 7.0, only to be followed by a decline at $P_H$ 7.4. On the acid side, the limiting concentration occurs below $P_H$ 2.8, whereas on the alkaline side it occurs about $P_H$ 8.8. Both examinations of prolonged incubation substantiate the data obtained from the first reading, the only difference being a gradual increase of germination with increase of incubation interval.

The same curve of germination is obtained at 31° C. The characteristic maximum occurs in the same culture as before, exhibiting the value $P_H$ 3.1; germination decreases with decrease in hydrogen ion concentration to the culture testing $P_H$ 7.0 where the minimum is obtained. A slight stimulation in germination is noted at $P_H$ 7.4, but no germination whatever is noticed with further decrease in hydrogen ion concentration. With an additional incubation of nine hours at the same temperature, germination relations remain practically the same. The range of germination is extended to $P_H$ 8.8, as compared with $P_H$ 7.4 at the first examination, and germination is extremely low at $P_H$ 7.0. Mycelial growth was so luxuriant in this series that it was

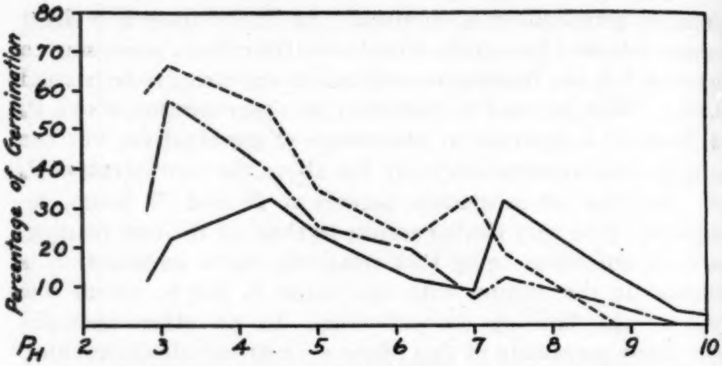



Fig. 2. *Penicillium cyclopium.* Graphic representation of the relation of germination to H ion concentration.

impossible to make a third reading. These relations are shown graphically in fig. 2.

An acid reaction decidedly favors spore germination of *Botrytis cinerea*, as seen by referring to table III.

TABLE III

BOTRYTIS CINEREA. AVERAGE PERCENTAGES OF SPORE GERMINATION IN M/5 MANNITE AT DIFFERENT TEMPERATURES AND AT VARIOUS HYDROGEN ION CONCENTRATIONS

| Temp. | Hrs. | Hydrogen ion concentration, $P_H$ | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 2.8 | 3.1 | 4.4 | 5.0 | 6.2 | 7.0 | 7.4 | 8.8 | 9.6 | 10.0+ |
| 22° C. | 6 | 70.0 | 86.6 | 63.4 | 52.6 | 48.2 | 22.3 | 0.0 | 0.0 | 0.0 | 0.0 |
| | 21 | 77.1 | 90.2 | 72.9 | 62.6 | 57.8 | 46.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 27° C. | 6 | 42.0 | 32.2 | 50.0 | 22.3 | 15.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| | 21 | 92.4 | 66.4 | 61.2 | 34.9 | 30.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |

Incubated at 22° C. no germination is obtained at $P_H$ 7.4; little germination is obtained at $P_H$ 7.0; and, with increasing hydrogen ion concentration, the germination quantities increase until a crest is reached in the culture with $P_H$ 3.1. With further increase of hydrogen ion concentration, there is a diminution in percentage of germination. The data obtained after an incubation period of 21 hours are very consistent with those of the

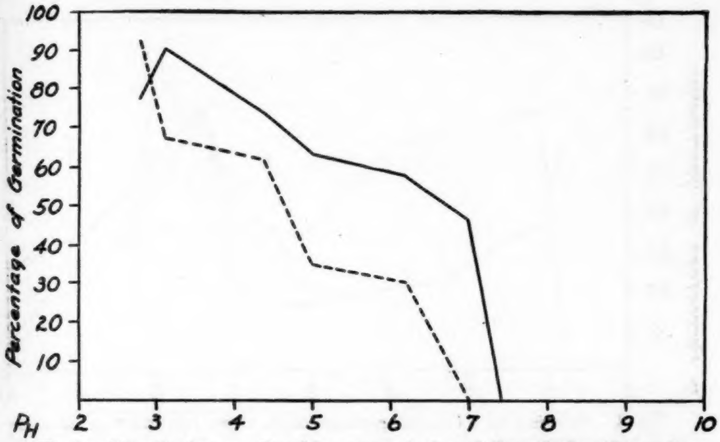



Fig. 3. *Botrytis cinerea.* Graphic representation of the relation of germination to H ion concentration.

6-hour period. Germination at 27° C. proved very similar to that at 22° C., except for the fact that the entire curve appears to have shifted one remove towards the acid side.

A series was made up and incubated at 31° C., but frequently the temperature went as high as 31.5° C. In no culture was there any sign of germination. This incidental datum is in accord with the results obtained by Duggar ('01). He found that a temperature of 32° C. was distinctly injurious to spores of *Botrytis.* Figure 3 exhibits the curves of germination at each of the successful temperatures.

TABLE IV

FUSARIUM SP. AVERAGE PERCENTAGES OF SPORE GERMINATION IN M/5 MANNITE AT DIFFERENT TEMPERATURES AND AT VARIOUS HYDROGEN ION CONCENTRATIONS

| Temp. | Hrs. | Hydrogen ion concentration, $P_H$ | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 2.8 | 3.1 | 4.4 | 5.0 | 6.2 | 7.0 | 7.4 | 8.8 | 9.6 | 10.0+ |
| 22° C. | 6 | 0.0 | 0.0 | 7.2 | 9.5 | 11.6 | 11.7 | 65.3 | 21.3 | 8.5 | 24.6 |
| | 20 | 57.8 | 38.0 | 21.9 | 20.7 | 16.0 | 20.1 | 67.7 | 36.6 | 27.7 | 26.9 |
| 27° C. | 6 | 0.0 | 9.7 | 31.0 | 45.4 | 18.0 | 23.3 | 50.0 | 23.7 | 5.1 | 0.0 |
| | 20 | 7.9 | 65.8 | 59.6 | 56.9 | 33.3 | 39.6 | 66.3 | 24.7 | 15.6 | 7.9 |

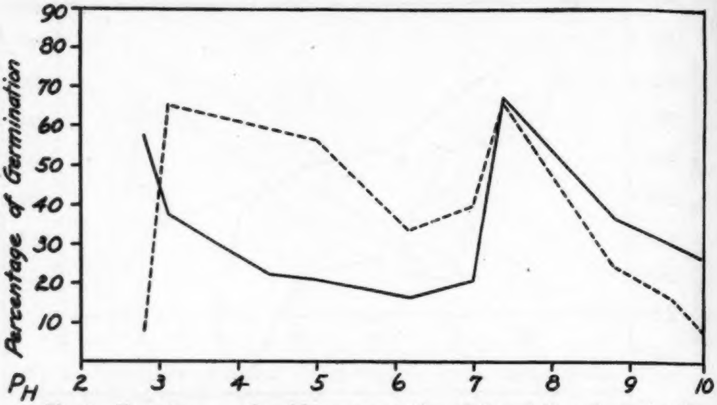



Fig. 4. *Fusarium* sp. Graphic representation of the relation of germination to H ion concentration.

Table IV shows that spores of *Fusarium* sp. are capable of germination over an extremely wide range of reaction. After incubation for 6 hours at 22° C. no germination is evident in the solution made to test $P_H$ 2.8, and the same is true with the culture testing $P_H$ 3.1, in very noticeable contrast with the results yielded by other forms.

Relatively small percentages of germination are obtained at $P_H$ 4.4, and germination gradually increases with decrease in hydrogen ion concentration until a very pronounced maximum is reached at $P_H$ 7.4. After this maximum, the curve declines only to rise suddenly at $P_H$ 10.0+. Upon further incubation, germination progresses rapidly in the extreme acid cultures, that testing $P_H$ 2.8 exhibiting maximum germination on the acid side. From this maximum, germination decreases to the culture possessing the exponent 6.2, rises slightly at $P_H$ 7.0, exhibits the usual maximum at $P_H$ 7.4, and then decreases with decrease in hydrogen ion concentration, the limiting concentration being beyond the culture testing $P_H$ 10.0+.

Examination after incubation for 6 hours at 27° C. shows that no germination is evident at $P_H$ 2.8, a fact noticed with the series incubated at 22° C. Slight germination occurs at $P_H$ 3.1 and increases to the culture exhibiting the value $P_H$ 5.0. Following the fall in germination at $P_H$ 6.2, there occurs a slight rise at $P_H$ 7.0 and the typical maximum at $P_H$ 7.4. After this crest is

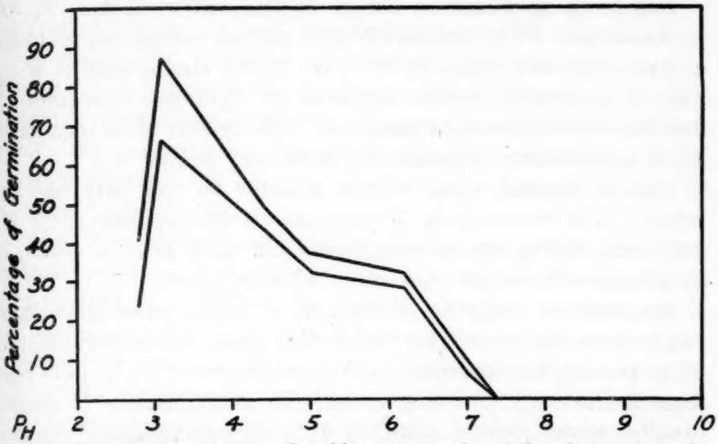



Fig. 5. *Lenzites saepiaria.* Graphic representation of the relation of germination to H ion concentration.

passed, germination decreases rapidly and appears to be totally inhibited at $P_H$ 10.0+.

With an additional incubation of fourteen hours, the order of germination on the acid side shifts considerably, while that on the alkaline side remains practically the same. Slight germination is obtained at $P_H$ 2.8, with the maximum on the acid side occurring at $P_H$ 3.1, and these are the only significant changes. A series was also incubated at 31° C., but on finding that two of the solutions had become contaminated, the data were discarded. In fig. 4 are shown the germination curves for the successful temperatures with this organism.

Very uniform data are obtained with the spores of *Lenzites saepiaria*, as shown by table v.

TABLE V

LENZITES SAEPIARIA. AVERAGE PERCENTAGES OF SPORE GERMINATION IN M/5 MANNITE AT 25° C. AND AT VARIOUS HYDROGEN ION CONCENTRATIONS

| Hrs. | Hydrogen ion concentration, $P_H$ | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 2.8 | 3.1 | 4.4 | 5.0 | 6.2 | 7.0 | 7.4 | 8.8 | 9.6 | 10.0+ |
| 18 | 23.6 | 66.3 | 43.3 | 32.1 | 28.3 | 6.5 | 0.0 | 0.0 | 0.0 | 0.0 |
| 30 | 40.4 | 87.4 | 49.3 | 37.4 | 32.2 | 8.6 | 0.0 | 0.0 | 0.0 | 0.0 |

Minimum germination occurs in the culture testing $P_H$ 7.0, and increases with increase of hydrogen ion concentration to the culture with the value $P_H$ 3.1. At $P_H$ 3.1 the maximum is obtained, and with further increase of hydrogen ions there is marked inhibition of germination. The results after incubation of 18 and 30 hours respectively, were very similar.

Due to limited time, it was possible to run only the one series. The curves in fig. 5 represent the germination at 25° C., one curve being constructed from the data after a period of incubation of 18 hours, the other after 30 hours.

Although no controls, as thought of in the usual sense, were run in the experiments reported in this paper, the cultures of each series possessing a hydrogen ion concentration of $P_H$ 7.0 have been regarded as such, it being considered that mannite in doubly distilled water gives a solution with an approximately neutral reaction. Such cultures then contained H and OH ions in equilibrium together with the other ions common to the cultures of the entire series, namely, sodium and phosphorus.

## Discussion

It is believed that the results here presented are sufficient materially to change the prevailing view as to the relation of spore germination to acid and alkaline media. Among the forms studied, germination is a process which is strikingly supported by a relatively high hydrogen ion concentration. In certain forms, secondary maxima may occur at approximately the neutral point, but only in one case among those studied, *Fusarium* sp., is the primary maximum near the neutral point or on the alkaline side. It is not necessary, of course, to assume that the hydrogen ion concentration most favorable for germination will also prove most favorable for the continued growth and development of the organism. Moreover, that is a problem outside of the present investigation.

Inasmuch as ordinary nutrient media for pathological and bacteriological work usually exhibit a hydrogen ion concentration approximately neutral or slightly acid, the data obtained from this investigation are further interesting in that they show that successively increasing concentrations of hydrogen ions, from

neutrality, favorably influence germination of the spores of *Aspergillus niger*, *Penicillium cyclopium*, *Botrytis cinerea*, *Fusarium* sp., and *Lenzites saepiaria* up to approximately $P_H$ 3.0. However, with increase of hydrogen ion concentration above this point, the germination quantities abruptly diminish. Some detailed discussion is however needed to compare these results with the work of others.

It has been shown that in the case of *Aspergillus niger*, maximum germination is obtained at $P_H$ 3.1, which expressed in terms of normality is N/1259. At $P_H$ 2.8, or N/631, germination is considerably better than at the neutral point, so that complete inhibition of germination must lie considerably higher than $P_H$ 2.8. Since the foregoing hydrogen ion concentrations have been expressed in terms of normality, it might be well to cite the concentrations of certain acids allowing normal or almost normal development of the spores of *Aspergillus flavus* in beet decoction, as determined by Clark ('99): HCl, N/64; $HNO_3$, N/64; $H_2SO_4$, N/128; acetic, N/64; monochloracetic, N/256; dichloracetic, N/128; trichloracetic, N/64; and HCN at N/8192. A mean of the limiting concentrations on the alkaline side for the various temperatures is $P_H$ 8.6, or N/251200, from which it appears that OH ions have the greater toxicity. In beet decoction, Clark found that N/16 KOH injured the spores of *Aspergillus flavus*, while N/8 was fatal; also that N/32 $NH_4OH$ inhibited germination, while N/16 was fatal. He concludes that the hydroxyl group, OH, is rather more toxic to the moulds studied than ionic H. However, as previously shown, the exact concentration of hydrogen ions in his cultures can not be calculated.

*Penicillium cyclopium* exhibits a relation to hydrogen ion concentration comparable with that of *Aspergillus niger*. Moreover, of all the forms which he studied, Clark found *Penicillium glaucum* the most resistant to acids and alkalis as well as to other poisons, the inhibiting concentrations on the whole being greater than those for *Aspergillus flavus*. Stevens' results indicated that *Penicillium crustaceum* is more resistant to poisons in aqueous solution than any of the other fungi studied by him. Growth occurred in N/50 HCl and $H_2SO_4$, while N/40 KOH and NaOH caused death. In my study, the rise in the germination

4

quantities at or about neutral is followed by a general decline. The limiting concentration on the alkaline side is about $P_H$ 10.0+, thus presenting a range of germination greater than that of *Aspergillus niger*.

From my results, *Botrytis cinerea* may be regarded either as very sensitive to an alkaline reaction in mannite solution or else as manifesting a certain dependence upon the stimulating effects of hydrogen ion concentration under such conditions. Not only is germination inhibited at $P_H$ 7.0–7.4, but the maximum, reached at $P_H$ 3.1–2.8, is equivalent to about N/1000 acid. The range of germination in this case is small.

*Lenzites saepiaria*, like *Botrytis cinerea*, proved very sensitive to an alkaline reaction, and, while the limiting concentration of the former on the acid side is somewhat lower than the latter, the two fungi are similar in behavior. Meacham ('18) obtained inhibition of growth of *Lenzites saepiaria* at about $P_H$ 1.7 in synthetic and malt-extract, and it is of interest to note that he frequently obtained a maximum of growth at about $P_H$ 3.0, which approaches very closely the hydrogen ion concentration of M/5 mannite which affords maximum germination, as reported in this paper.

Of the forms studied, *Fusarium* sp. is the only one that responded favorably to an alkaline medium. Moreover, this form exhibits about the widest range of germination, yet the behavior was variable and discordant results were not infrequent.

## Conclusions

Under the conditions described and as far as the experiments have gone, the following conclusions may be drawn:

(1) In a culture solution consisting of M/5 mannite, phosphoric acid, and sodium hydroxide, successively increasing concentrations of hydrogen ions from neutral or approximately neutral to $P_H$ 3.1–2.8 favorably influence germination of the spores of *Aspergillus niger*, *Penicillium cyclopium*, *Botrytis cinerea*, *Fusarium* sp., and *Lenzites saepiaria*.

(2) The range of germination and the magnitude of the germination quantities as influenced by hydrogen ion concentration in the solution mentioned depend upon the organism, germina-

tion being obtained with the following concentrations, inclusive: *Aspergillus niger*, $P_H$ 2.8–8.8; *Penicillium cyclopium*, 2.8–10.0+; *Botrytis cinerea*, 2.8–7.0; *Fusarium* sp., 2.8–10.0+; and *Lenzites saepiaria*, 2.8–7.0.

(3) It is not until a hydrogen ion concentration of $P_H$ 2.8 or above is reached that inhibition of germination of the forms studied is noticed.

(4) *Aspergillus niger*, *Penicillium cyclopium*, *Botrytis cinerea*, and *Lenzites saepiaria* show a maximum of germination in the medium employed at $P_H$ 2.8–3.1; *Fusarium* sp. exhibits a secondary maximum at this concentration.

(5) *Fusarium* sp. gives a pronounced maximum of germination at $P_H$ 7.4, and *Penicillium cyclopium* exhibits a minor secondary maximum at $P_H$ 7.0–7.4.

(6) For equal removes from the neutral point, $OH'$ ions appear to be relatively more toxic to the spores studied than H ions.

(7) With increase in length of intervals of incubation, the relations of germination to hydrogen ion concentration remain practically the same.

(8) The curves of germination for any organism are practically identical, whether incubated at 22° C., 27° C., or 31° C.

The writer takes pleasure in acknowledging his indebtedness and extending his sincere thanks to Dr. B. M. Duggar for suggesting and directing the problem and for kindly criticism and advice; and to Dr. George T. Moore for the privileges and facilities of the Missouri Botanical Garden.

*Graduate Laboratory, Missouri Botanical Garden.*

## Bibliography

Ayers, S. H. ('16). Hydrogen ion concentrations in cultures of streptococci. [Jour. Bact. 1: 84–85. 1916.]

Brooks, C. ('06). Temperature and toxic action. Bot. Gaz. 42: 359–375. 1906.

Clark, J. F. ('99). On the toxic effect of deleterious agents on the germination and development of certain filamentous fungi. *Ibid.* 28: 289–327, 378–404. 1899.

Clark, W. M., and Lubs, H. A. ('17). The colorimetric determination of hydrogen ion concentration and its application in bacteriology. Jour. Bact. 2: 1–34, 109–136, 191–236. *f. 1–8.* 1917.

Duggar, B. M. ('01). Physiological studies with reference to the germination of certain fungous spores. Bot. Gaz. **31**: 38–66. 1901.

———, Severy, J. W., and Schmitz, H. ('17). Studies in the physiology of the fungi. IV. The growth of certain fungi in plant decoctions. Ann. Mo. Bot. Gard. **4**: 165–173. *f. 1–4.* 1917.

Ferguson, Margaret C. ('02). A preliminary study of the germination of the spores of Agaricus campestris and other basidiomycetous fungi. U. S. Dept. Agr., Bur. Pl. Ind. Bul. 16: 1–40. *pl. 1–3.* 1902.

Fred, E. B., and Davenport, A. ('18). Influence of reaction on nitrogen-assimilating bacteria. Jour. Agr. Res. **14**: 317–336. *f. 1.* 1918.

———, and Loomis, N. E. ('17). Influence of hydrogen-ion concentration of medium on the reproduction of alfalfa bacteria. Jour. Bact. **2**: 629–633. 1917.

Gruzit, O. M. ('17). The effect of some acids and alkalies on soil bacteria in the soil solution. Soil Sci. **3**: 289–295. *f. 1–2.* 1917.

Krönig, B., und Paul, Th. ('97). Die chemischen Grundlagen der Lehre von der Giftwirkung und Desinfection. Zeitschr. f. Hyg. und Infect. **25**: 1–112. *pl. 1.* 1897.

Meacham, M. R. ('18). Note upon the hydrogen ion concentration necessary to inhibit the growth of four wood-destroying fungi. Science N. S. **48**: 499–500. *f. 1.* 1918.

Morgan, J. F., and Gruzit, O. M. ('16). Reaction of the soil solution as an index of biological changes in the soil. [Jour. Bact. **1**: 97. 1916.]

Salter, R. C. ('16). The behavior of legume bacteria in acid and alkaline media. Iowa Acad. Sci., Proc. **23**: 309–312. *pl. 8.* 1916.

Spaulding, P. ('11). The timber rot caused by Lenzites saepiaria. U. S. Dept. Agr., Bur. Pl. Ind. Bul. **214**: 1–46. *pl. 1–4.* 1911.

Stevens, F. L. ('98). The effect of aqueous solutions upon the germination of fungous spores. Bot. Gaz. **26**: 377–406. 1898.

Taylor, K. ('17). Specificity of antiseptics. The Lancet **1**: 294–297. 1917.

Wolf, C. G. L., and Harris, J. E. G. ('17). Contributions to the biochemistry of pathogenic anaerobes. III. The effect of acids on the growth of Bacillus Welchii (B. perfringens) and Bacillus sporogenes (Metchnikoff). Biochem. Jour. **11**: 213–245. *f. 1–8.* 1917.

Wright, J. H. ('17). The importance of uniform culture media in the bacteriological examination of disinfectants. Jour. Bact. **2**: 315–346. 1917.

Zeller, S. M. ('16). Studies in the physiology of the fungi. II. Lenzites saepiaria Fries, with special reference to enzyme activity. Ann. Mo. Bot. Gard. **3**: 439–512. *pl. 8–9.* 1916.

# DIASTASE ACTIVITY IN RELATION TO STAGE OF DEVELOPMENT AND CARBOHYDRATE CONTENT OF THE TUBER OF SOLANUM TUBEROSUM[1]

RUPERT A. McGINTY

*Formerly Rufus J. Lackland Fellow in the Henry Shaw School of Botany of Washington University*

The effect upon enzyme activity of various factors, such as temperature, light, and different concentrations of salts, acids, and alkalis, has been studied in considerable detail, and the behavior of the enzymes, with respect to these factors, has thrown much light upon physiological processes. One phase of this question, however, has been touched upon only to a slight extent, that is, the relation of enzyme activity to the various stages of growth of plant organs. For this reason, it was thought that a study of the activity of the enzyme diastase, in relation to tuber growth in *Solanum tuberosum*, might prove profitable. At the same time, it was deemed of interest to follow the changes in starch and sugar content at the different stages, and thus determine whether any correlation exists between diastase activity, growth, and carbohydrate content.

## Review of Literature

The fundamental importance of enzymes in the processes of metabolism has resulted in a voluminous literature on the subject, but a survey of this literature reveals only a very few papers which have a direct bearing on the phase of the subject considered in the present instance. These papers are briefly reviewed below. A larger number have a more or less indirect bearing upon the topic here discussed, and some of these will also be considered.

Probably the first observations on the presence of diastase in the potato were made by Payen and Persoz ('33). They found

[1] An investigation carried out at the Missouri Botanical Garden in the Graduate Laboratory of the Henry Shaw School of Botany of Washington University, and submitted as a thesis in partial fulfillment of the requirements for the degree of master of arts in the Henry Shaw School of Botany of Washington University.

it to be present in the tubers during growth, and also detected its presence in oats, wheat, maize, and rice during germination. They were the first investigators to prepare the enzyme from the extracts of germinated grain, extracting the latter with water and precipitating the ferment by means of alcohol. To this alcoholic precipitate they applied the term diastase, which has persisted since that time. Subsequent investigation showed diastase to be present not only in tissues in which starch normally occurs as a storage product, but also in some where the reserve materials are stored as sugar. Thus, in 1878, Baranetzky found diastase present in the roots of carrots and turnips, which contain no starch. He also found it in the leaves and stems of several plants and in potato tubers. From this evidence, he suggested that diastase was probably universally present in living cells, and later work has practically confirmed this opinion.

Müller-Thurgau ('82) showed that under certain conditions the amount of cupric-reducing substances in leaves increased at the expense of the starch present. He also found ('85) that exposure of potato tubers to a temperature of 0° C. for a month resulted in an accumulation of sugars, with a corresponding loss of starch. Contrary to popular opinion, it was determined by him that no sugar was formed in potatoes which were actually frozen. He has also found that when potatoes which had become sweet by exposure to low temperatures were placed at a temperature of 8–10° C. the sugar disappeared. Müller-Thurgau considered these phenomena to be due to an enzymic process, which, while more rapid at high temperatures, occurs also at low temperatures. According to his ideas, the lessened respiration at low temperatures, entailing the use of less sugar, together with an inhibition of re-formation of starch from sugar—which re-formation takes place rapidly at high temperatures—allows the sugars to accumulate when potatoes are kept at 0° C.

Brasse ('84) is considered by Brown and Morris ('93) to have been the first to prove conclusively the presence of diastase in leaves. He examined the leaves of the potato, dahlia, beet, tobacco, and some other plants, and measured the rate of the activity of the diastase obtained from extracts by precipitation with alcohol. This product was allowed to act upon starch

paste, the rate of activity being determined by the cupric-reducing power of the solution. He used chloroform to inhibit the growth of microörganisms during the time the enzyme was allowed to act, being one of the first to pay attention to this point.

The relative amounts of different carbohydrates in plants often bear some relation to enzyme activity; therefore, the early work of Hungerbühler ('86), who estimated the carbohydrates at different times in growing potatoes, is of interest here. The following figures give the amounts of reducing sugar, invert sugar, and starch, expressed in percentages of the dry weight, found by him in the tubers at different times:

| | June 23 | June 30 | July 7 |
|---|---|---|---|
| Reducing sugars | 6.4 | .32 | .72 |
| Invert sugar | | 4.50 | 4.69 |
| Starch | 56.7 | 61.30 | 66.30 |

Schulze and Seliwanoff ('88) showed the amount of sucrose in immature potatoes to be especially high. Brown and Morris ('90) determined the amounts of diastase present in barley grains at three stages in their development. The results were as follows:

| | Relative diastatic activity |
|---|---|
| Endosperm half developed | 4.4 |
| Endosperm two-thirds developed | 7.8 |
| Endosperm completely developed | 9.7 |

These results are in line with the idea that the appearance and increase of the enzyme are related to the formation and nutrition of the embryo.

The same authors studied the germination of barley grains and found the amount of diastase to increase markedly during the process. The diastatic activity of the barley embryos after different periods of germination was found to be as follows:

| | Relative diastatic activity |
|---|---|
| Embryos, dissected from grains which had soaked in water for 24 hours | Trace only |
| Embryos from same barley, but germinated for 3 days on 5 per cent gelatin. Diastatic activity of embryos plus substrate | .1186 |
| Same, except germinated for 4 days | .1634 |
| Same, except germinated for 6 days | .2432 |

There has, in general, been agreement among investigators as to the occurrence of diastase in plant tissues, but a notable exception in this respect was Wortmann ('90), who concluded from his experiments that the dissolution of starch in plants is brought about directly by the protoplasm and independently of the diastase present. This conclusion was based on experiments made by him which showed diastase to be absent from most leaves, and to occur in others in such small quantity and in such a state as to exhibit only very feeble activity. Even in those cases where this slight activity was manifested it would not, according to him, account for the transformation of starch which actually takes place.

It is of interest in this connection to note that Schimper ('85) had found that starch-free leaves of *Allium* were much less diastatically active than the leaves of *Tropaeolum*, which contain much starch. The comparatively recent work of Bradley and Kellersberger ('12) in this particular should also be mentioned. They found that the leaves of many different species of plants varied greatly in their diastatic content, some, such as the bayberry, onion, and leek, giving so little reaction as to make the presence of the enzyme doubtful.

Brown and Morris ('93) could not agree with the theory of Wortmann, referred to above, that the protoplasm and not an enzyme is directly responsible for the transformation of starch in leaves, but believed, as did Baranetzky, that diastase is universally present in these organs, and that dissolution of starch is brought about by it. They criticized Wortmann's work, chiefly on the ground that he used the clear filtered leaf extracts in his experiments and did not take into consideration the tannins of the leaves, which they found to interfere greatly with the extraction and activity of the enzyme. The clear filtered leaf extract was found by them to possess much less activity than the same extract before filtering.

This work of Brown and Morris is perhaps the most comprehensive and conclusive that has been carried out upon the presence of diastase in plants. They determined the activity of the enzyme in all cases by the addition of .5 gm. of finely powdered, air-dried leaves to 50 cc. of a 2 per cent starch solution

containing 5 cc. of chloroform per liter as preservative. The digestion was carried on for 48 hours, after which the cupric-reducing power of the solution was determined. Their experiments showed diastase to be present in all leaves examined and that it always possessed a decided activity, sufficient even in the cases of the lowest diastase content to transform more starch than the leaves ever contain at one time. These investigators may therefore be considered to have added further evidence in support of the view of Baranetzky, referred to above, that all living cells contain diastase. Only a few facts remain which render it somewhat doubtful. Duggar and Davis ('14), in this laboratory, were unable to demonstrate the presence of the enzyme in *Fucus*, and Bradley and Kellersberger ('12) were doubtful of its presence in such plants as onions, leeks, and certain mushrooms. Failure to find diastase or invertase in the flesh of ripe apples is also reported by Thatcher ('15).

The comparative tests of diastatic activity made by Brown and Morris, referred to above, showed it to be especially high in leguminous plants, while the members of the *Liliaceae* proved to be poor in the enzyme, corresponding with the small amount of starch they contain. To give an idea as to the relative diastatic activity of the leaves of different species, as determined by them, the following plants are selected from the large number reported:

| Plant | Relative diastatic activity |
|---|---|
| *Pisum sativum* | 240.30 |
| *Trifolium pratense* | 89.66 |
| *Solanum tuberosum* | 8.16 |
| *Lycopersicum esculentum* | 6.57 |
| *Allium Cepa* | 3.76 |
| *Hydrocharis Morsus-ranae* | .27 |

Attention is directed to the position of the potato in this table, which indicates a comparatively low diastase content.

When the diastatic activity of a set of half-leaves gathered at one time during the day was compared with that of the corresponding half-leaves picked at another time during the same day, it was found to vary considerably. They consider the figures obtained in the last-mentioned case to indicate that the condi-

tions favorable for starch formation are not favorable for enzyme activity and vice versa, since the enzyme activity was much higher at night than during the day. These findings led Green ('97) to investigate the effect of light upon diastase. He found the activity of the enzyme to be considerably decreased by exposure to sunlight, the deleterious effect varying with the intensity of the illumination.

Brown and Morris also demonstrated that diastase, from certain leaves at least, will hydrolyze solid starch, a point disputed by Wortmann. They concluded, too, that their experiments established almost beyond doubt, that, while protoplasm may exert some influence upon the action of diastase upon starch in the early stages of the action, the dissolution of starch is mainly brought about by the enzyme diastase.

In addition to their investigations of diastase in leaves, these authors also worked upon the carbohydrate content of leaves. They concluded that cane sugar, dextrose, levulose, and maltose are present in leaves, but failed to find any pentoses. The amounts of these sugars were found by them to increase in the light and to decrease in the dark, with the exception of levulose, which in many cases followed a reverse procedure. The increase in levulose in the dark was attributed by them to the inversion of cane sugar.

Finally, these authors believed "starvation" of the cell to induce the dissolution of starch through the formation of diastase, and concluded that of the carbohydrates disappearing from leaves in the dark, maltose and levulose contribute most to the respiratory requirements of the cells. This idea of diastase secretion being induced by starvation of the cell is concurred in by Effront ('02, p. 139), who says: "The secretion [of amylase by barley embryos] is always abundant when the germ is found in poor nutritive condition, and it is checked as soon as an assimilable substance appears."

The work of Meyer ('85) was briefly reviewed by Brown and Morris in connection with their work on the carbohydrates of the leaf. Meyer attempted to ascertain if the leaves of such plants as *Allium Cepa,* which store no starch, contain any other cupric-reducing carbohydrates comparable in amount with the

starch of other leaves. He found that plants which store starch abundantly contain comparatively little soluble reducing and non-reducing carbohydrates in their leaves, while others, such as *Allium Cepa*, *Iris germanica*, and *Gentiana lutea*, which store no starch, accumulate relatively large quantities of soluble reducing substances in their leaves. These reducing substances, according to him, appear to be regulated by the same laws as starch in relation to their appearance and disappearance in light and darkness.

The formation and distribution of diastase in the potato tuber was investigated by Prunet ('92), who observed during germination that diastase activity was greater toward the "seed end" of the potato than elsewhere. This corresponds with the greater development of shoots from that region. He found also a correspondence between the sugars present in the different portions of the tuber and the amount of diastase.

Green ('99) states that in some experiments carried on by him in 1893 he found diastase to be present in the pollen of many plants. The amount usually increased considerably at the onset of germination, and appears, it is stated, "to accompany the tube as the latter elongates, which suggests a formation not only in the grain but in its tube also." As the pollen loses with age the power of germination, it also loses its diastase, which is an indication of the part played by the enzyme in germination. This, Green suggests, is comparable with the influence exerted by diastase in the germination of the potato.

The effect of enzymes upon seed germination was the subject of some study by Waugh ('98). Employing solutions of diastase and some other enzymes, he found that the percentage of germination of old tomato seed was considerably increased, in the case of diastase, where the seeds were soaked in the solution for several hours. Taka-diastase gave somewhat higher, but not as uniformly favorable, results as malt diastase. Trypsin also gave good results in some cases.

The work of Keitt ('11) on the formation of sugars and starch in the sweet-potato led him to believe that in the very immature tubers the sugar might be present either as glucose or sucrose, dependent perhaps upon meteorological conditions. He found

the total sugars to decrease gradually as the plants matured, while the starch apparently increased until the vines were killed by frost, at which time it was at its maximum. Killing of the vines by frost was followed by a breaking down of starch with a tendency toward the formation of sucrose. The following is a typical example of his analyses, in which figures are given for the small immature tubers harvested on August 28, and for successive stages until the potatoes were mature:

| Date | Starch | Glucose | Sucrose |
|---|---|---|---|
| Aug. 28 | 17.88 | .61 | 4.18 |
| Sept. 7 | 14.91 | 1.58 | 1.82 |
| Sept. 18 | 17.61 | 1.88 | 1.91 |
| Sept. 29 | 19.92 | 2.30 | 1.43 |
| Nov. 18 | 17.82 | 1.53 | 2.35 |

(A freeze occurred on Nov. 6 which killed the vines.)

It has been generally assumed that transformations brought about through the agency of enzymes are reversible; that is, diastase which hydrolyzes starch to maltose also acts as the synthesizing agent in the production of starch. The hydrolytic processes have, of course, received most attention, but have not been exclusively investigated. The synthetic aspect of the problem was attacked by Bradley and Kellersberger ('12). In view of the results obtained, they were unwilling to make any general deductions as to the relation between the diastatic activity of a tissue and its starch-storing function, though their determinations seemed to give considerable support to the view that the enzyme is the synthesizing agent. One reason for their hesitancy in formulating a conclusion was their inability to explain the fact that fresh mushroom tissues, rich in a glycogen-like carbohydrate, when allowed to act upon a soluble starch solution for five days, showed no evidence of diastase activity, while the same tissues ground up with alcohol, washed with ether, and dried, developed considerable activity. They thought this might be explained by the fact that the treatment activated a proenzyme, but if such were the case, they could not understand why in a rapidly growing mushroom in which the glycogen transfer must necessarily be rapid diastase should not be present, if that enzyme is necessary for synthesis.

Some other facts also introduced an element of doubt into the matter. Beets and mangels, which store their carbohydrates as sugar instead of starch, were found to possess highly diastatic leaves, while the roots contained none of the enzyme. No trace of starch could be found in roots of the radish, yet it was one of the most diastatically active tissues studied. Likewise the potato tuber, which is, of course, particularly rich in starch, proved to be poor in diastase. The leaves of different species of plants were found by them to vary greatly in diastase content. The enzyme appeared most abundant in leaves where, to quote them, "starch is never stored permanently, but where it may be found in small amounts during photosynthesis." These statements do not agree very well with that made by Haas and Hill ('17, p. 369), which is as follows: "The amount of diastase is always greater in starch leaves than in sugar leaves, and the same holds for insolated leaves containing much starch, as compared with shaded leaves containing little or no starch."

In studying this problem of carbohydrate synthesis in plants, Bradley and Kellersberger made some determinations of diastatic activity which are of interest in connection with the present problem, and a few of these are given below. The figures have been recalculated on the basis of 100 which represents the greatest activity reported.

| Plant | Relative diastatic activity | Amount of starch content |
|---|---|---|
| Pea, seeds, medium | 100.0 | Medium |
| Pea, seeds, mature | 50.0 | Abundant |
| Corn, seeds, young | 10.0 | Medium |
| Corn, seeds, medium | 6.7 | Abundant |
| Corn, seeds, mature | 7.7 | Abundant |
| Corn, cob, young | 7.7 | Small |
| Corn, cob, mature | 7.7 | Small |
| Corn, husk, young | 25.0 | None |
| Corn, husk, mature | 8.3 | None |
| Potato, root, medium | 2.8 | Some dextrin |
| Potato, tuber, medium | 2.9 | Very abundant |
| Potato, leaf, medium | 75.0 | None* |

* Potato leaves generally contain starch, but in this case they were obtained in the early morning before photosynthesis had taken place to any extent.

The diastatic activity of both potato and onion leaves was determined by Brown and Morris ('93) and by Bradley and Kellersberger. Thus it is possible to compare, by means of the values given for onion leaves, the activities of the potato leaves as found in the two cases. Different units, of course, are used to express the enzyme activity in the two cases.

| Determination made by | Relative diastatic activity Potato leaves | Onion leaves |
|---|---|---|
| Brown and Morris | 8.2 | 3.80 |
| Bradley and Kellersberger | 75.0 | 1.05 |

It is seen from the above that the relative activity in the case of the potato, as found by Bradley and Kellersberger, was much higher than that found by Brown and Morris. This is doubtless partly due to the fact that the former authors made their determinations by means of the cupric-reducing power of the solutions, while the latter employed the iodine reaction. It illustrates the wide difference that often occurs in the results obtained by different workers along this line. In this case, however, the difference is not one of direction, but of degree only.

The physiological changes taking place in the potato during its rest period have been studied to a considerable degree by Appleman ('11, '16). He found, as did Müller-Thurgau and others, that glucose and sucrose accumulate in potatoes which are stored at 0° C. Diastase activity was also greater at the end of 2 and 4 weeks in the cold-storage potatoes than in those stored at room temperature, but at the end of 6 weeks practically no difference could be detected, as, he explains, this was near the end of the rest period of the variety used in this work. Appleman ('11) says that "the increased diastatic activity is probably due to greater activation of zymogen by free acids which are liberated by the greater permeability of protoplasmic membranes at low temperatures."

It is well known that potatoes will not germinate for several weeks after being harvested, apparently because certain changes known as "after-ripening" must first take place. In attempting to determine the nature of these changes, Appleman ('16) reached the conclusion that carbohydrate transformations in the potato tuber during its rest period must not be considered

after-ripening processes, but simply due to, and dependent upon, changing temperature. He did not find an increase at the time of sprouting in the reducing or total sugars in the case of potatoes which, since harvesting, had been stored under growing conditions; nor was there any difference in the sugar content of the seed and stem ends of the tubers at the beginning of sprouting. The diastase activity, he states, was uniformly greater in the extract from the seed end, but there was no appreciable increase in diastatic activity in either end during the rest period, in spite of the fact that sprouting begins much earlier in the seed end. From this evidence, the conclusion is reached that the "cessation of the rest period is not due to a gradual increase in diastatic activity."

Appleman ('16) found further that sprouting could be brought about at any time during the rest period by removing the skins of the tubers. There was still earlier sprouting in tubers which were cut in half transversely, those buds near the exposed surface starting first. Subdued light and a treatment consisting of wrapping the potatoes with cotton saturated with hydrogen peroxide were both effective in shortening the rest period in new tubers with skins not highly suberized. The author believed the shortening of the rest period in all these cases to be correlated, not with water absorption, but with increased absorption of oxygen.

In his studies of the rest period of plants, Howard ('15) found that when certain agents, such as etherization, desiccation, warm water bath, etc., were used to break the rest period, the diastatic activity of the treated tissues was increased, such increase agreeing in each case with the extent to which the treatment broke the rest period. Corresponding with this increased diastase activity, the amounts of soluble reducing sugars were also found to increase within 24 hours after application of the treatment, provided the treatment was applied during the early winter. Treatments given later in the season were found to have very little effect.

The work of Howard is confirmed to some extent by that of Bonns ('18) in this laboratory. The latter, in studying the effect of etherization upon enzyme activity in corms of *Gladiolus*,

found increased hydrolysis of starch by the enzyme, following treatment with the anaesthetic.

Butler ('13) observed, by means of the coloration produced in slices of tubers by boiling them in Fehling's solution, and also by analyses, the sugar content in potatoes during the rest period and at the time of sprouting. He makes the statement that the accumulation of sugar in the tubers has no particular physiological significance as regards germination. He calls attention to the fact that tubers which germinate at a relatively low temperature contain more sugar than those which germinate at relatively high temperatures, and also that those which are allowed to sprout in the soil have a higher sugar content than those sprouted in the cellar. Certain sprouting potatoes examined by him were found to contain no sugar, though the sprouts themselves contained a considerable amount. The same potatoes, after being stored in an ice chest for 20 days, were found to have accumulated a considerable amount of sugar, while the sugar in the sprouts apparently remained constant. At the same time potatoes taken from storage where the temperature had not fallen below 6° C. showed considerable sugar in both tuber and sprouts. His illustrations show that stored potatoes kept under similar conditions were quite variable as to sugar content, some not containing sugar at all or in the cortex only, others in the medulla only, and still others in both cortex and medulla.

According to some further results obtained by Butler, there is less sugar in the vicinity of sprouting eyes than elsewhere, and by analyses he found the sugar content of the seed ends of sprouting potatoes to be small, as a rule, and to increase toward the stem end. This is at variance with the results of Prunet ('92), who found the greater amount of sugar in the seed end of the tuber, and also with those of Appleman ('16), who found no appreciable difference in the sugar content of the opposite ends of those potatoes examined by him.

Butler believes that the greater metabolic activity at the seed end of the tuber, both during the rest period and at germination, is unfavorable for an accumulation of sugar at that point, and concludes from his data that there is "little if any translocation from remote to budding parts, even in germinating potatoes."

The author states, finally, that since the distribution of sugar in resting and germinating potatoes is not essentially different, its appearance in quantity at the time of germination should be ascribed, partly at least, to metabolic changes induced by other agencies.

Doby and Bodnar ('15) investigated diastatic activity in tubers from healthy potato plants as compared with that in tubers from plants affected with leaf roll. They found the absolute value of the diastase (diastase plus zymogen) to be the same in healthy and diseased potatoes, but, in general, more zymogen was found present in healthy than in diseased tubers. These workers also investigated the presence of zymogen in the extracted juice of potatoes. They observed that the diastatic activity of the juice increased upon standing, which fact they attribute to the rapid conversion, in the extracted juice, of zymogen into amylase. Fresh juice, which was quite active to begin with, did not increase to the same extent as did other juice in which diastase was less active in the beginning. Less zymogen was found to be present in tubers in the middle of the rest period than at other times. It began to increase during the first half of January, and a corresponding increase in diastatic activity was observed at the same time. In the spring only small amounts of zymogen were found, since at that time most of it had been changed into active diastase.

It was believed by these investigators that the diastatic activity of fresh juice depends chiefly upon the stage of development of the tuber, but that it may also be affected by the kind of potato used and by climatic and cultural conditions during growth. The stage of the rest period must also be taken into consideration when determinations are made during that time. Enzyme activity was found to be almost entirely independent of the size of the tubers. This calls attention to the necessity for the consideration of these factors in relative diastatic activity determinations, and emphasizes the importance of a uniform procedure as regards the time that elapses between the different operations necessary in such determinations, as well as the importance of using material, the different stages of which have developed under known and comparable conditions.

One of the last-mentioned authors, Doby ('14), carried out some experiments to determine the effect of the different sugars upon the amylase of potatoes. From the results obtained, he concluded that the action of the enzyme is inhibited by the sugars in the following order, maltose being most effective,—maltose, glucose, fructose, arabinose, galactose, mannose.

He further concluded from his experiments that it is quite probable that the action of amylase in a natural enzyme system is restricted, in the first place by the decomposition products of the substrate, and in the second place by other sugars which may be present.

Since maltose is the final product in the hydrolysis of starch by amylase, it might seem that the activity of the enzyme would soon be checked by the accumulation of this sugar, but Davis, Daish, and Sawyer ('16), in their extensive work upon the formation and translocation of carbohydrates in plants, found that maltose was immediately transformed into hexose by the maltase present, so that there is only a trace of this sugar, in leaves at least, at any one time. Moreover, Norris ('14) states, as a result of experiments upon the factors influencing hydrolysis of glycogen by diastase, that while the mixed products of hydrolysis have a marked retarding influence on the velocity of the reaction, maltose alone has very little effect.

In studying the effect of activators upon the diastase of potatoes, Doby and Bodnar ('15) found, among other things, that the boiled juice exerted a considerable activating influence. Effront ('02) made a similar observation in regard to malt diastase.

Carbohydrate transformations in the sweet-potato resemble to a degree those in *Solanum tuberosum*. Hasselbring and Hawkins ('15, '15a), and Hasselbring ('18), from their studies on this subject, reached the conclusion that, while sweet-potatoes contain only very small amounts of sugar during growth, the transformation of starch into sugar begins immediately after harvesting or upon the killing of the vines by frost. The same changes were found to occur whether the tubers were stored in cellars or remained in the ground. These changes involved, according to the authors, the transformation of starch to reducing sugars

and the subsequent synthesis of cane sugar from these. The formation of sugars was observed to be rapid at a high temperature (30° C.), more gradual at temperatures of 11.7–16.7° C., while lowering the temperature to 4° C. again accelerated the process.

Hasselbring and Hawkins ('15) also found, in the course of further experiments, that the carbohydrate content of the two halves of a potato, which had been split longitudinally, was not the same in all cases. While the analyses of the two halves of a freshly dug tuber agreed closely, differences of from 1 to 16 per cent were found in corresponding halves of tubers which had been kept for a time. These differences were found regardless of the temperature at which the potatoes had been kept. These authors consider their work to confirm and extend the investigations of Keitt, referred to previously.

The extensive work of Davis, Daish, and Sawyer ('16) on the carbohydrates of the mangold leaf, and that of Davis and Sawyer ('16) on the carbohydrates of potato leaves are perhaps the most recent contributions along this line. These investigators not only secured much valuable information regarding distribution of sugars and starch at different hours of the day in the leaves mentioned, but also developed methods for the quantitative determination of these substances in plant material. These methods, while they may, according to Jörgensen and Stiles ('17), be subject to certain errors, are doubtless the most reliable to be found at the present time.

Some of the results obtained in this work were as follows: (1) Starch was found to be entirely absent from mangold leaves except in the early stages of growth, and maltose was never present, either in the leaves, stalks, or midribs, at any time of the day or night. (2) Sucrose was present in the leaf in excess of hexoses in the early stages of growth, but later, when sugar was being stored in the root, the hexoses predominated. The latter sugars were also more abundant than sucrose in the midribs and leaf-stalks. Sucrose was therefore concluded to be the first sugar formed in photosynthesis, being transformed into hexoses for the purpose of translocation. Pentoses were found to form a small proportion of the sugars in the tissues. (3) The

hydrolysis of starch was believed to be effected by a mixture of enzymes, containing maltase in relative excess, so that the transformation of starch to dextrose was complete.

The conclusions in regard to sucrose are in agreement with the views of Brown and Morris ('93). The absence of maltose, however, is contrary to the findings of the latter authors, and is due, according to Davis, Daish, and Sawyer, to the fact that enzyme activity was not checked promptly in the material examined by them. Disagreement also exists in regard to pentoses, Brown and Morris not finding these sugars present.

## Materials and Methods

Potatoes of the Irish cobbler variety were used in this work. They were grown on a bench in the greenhouse, and, as is usually the case under such conditions, the mature tubers were small, the largest being only 6 cm. in diameter. It was the aim at the outset to make analyses of the tubers for enzyme activity, reducing sugars, sucrose, and starch, beginning when the young potatoes had attained a diameter of 1 cm., and making later determinations corresponding with each increase of 1 cm. in diameter until maturity. This plan has been followed with the exception that it was not possible to obtain sufficient tubers of the first size (1 cm. in diameter) for the determinations of both diastase activity and carbohydrate content, but enough were found for the enzyme activity determinations. Some analyses were also made of seed potatoes.

Upon reaching the proper size the potatoes were harvested, washed free from all particles of dirt, and dried with a towel, after which the analysis of the material was begun without delay. These and all other operations were carried out as quickly as possible, so as to avoid errors which might be due to delay in handling.

This procedure may be objected to by some on the ground that size of the potato tuber is not necessarily correlated with stage of development. In these experiments, however, the first four sizes (1–4 cm. in diameter, inclusive) were all dug at the same time, while the vines were still actively growing, and the 5- and 6-cm. sizes were secured about two weeks later. All

these tubers, except the largest size, which was practically mature, were still in an active, growing condition, and it is believed that they really represented not only different sizes but also different stages of development. This should be especially true in the case of the enzyme activity determinations, as it was necessary in making these to use a rather large number of the tubers to obtain the juice needed, thus providing a composite sample.

*Diastase activity.*—Two or three methods of obtaining the enzyme were tried before a satisfactory one was found. When the potatoes were sliced and dried by means of alcohol and acetone, according to the method used by Davis ('15), and then finely ground in a mortar, extracted with water, and the enzyme precipitated with 95 per cent alcohol, the diastase so obtained was apparently not active enough to make the method satisfactory.

A method whereby the enzyme was precipitated by alcohol from the pure juice of the potato was then tried. The juice was obtained by quickly grating the tubers, grinding the material in a mortar with carborundum, squeezing the juice out by means of a tourniquet of cheese-cloth, and finally filtering through asbestos and filter-paper. The filtering was done in order to remove the starch present. The enzyme obtained from this juice by precipitation with alcohol apparently possessed no activity whatever, and it was finally decided to use the juice itself, as preliminary tests had shown it to be quite active diastatically. The fact that the juice of the potato has considerable activating influence upon the diastase, as found by Doby ('14), was confirmed, and this no doubt accounts for the inactivity of the alcohol-precipitated enzyme.

In determining diastatic activity, 20 cc. of the fresh juice were added to 100 cc. of a .25 per cent soluble starch solution in a 250-cc. Erlenmeyer flask, 2 cc. of toluol added, and the flasks placed in the incubator and kept at 45° C. for 12 hours. At the end of this time the flasks were placed in boiling water for 10 minutes to kill the enzyme.

At the same time checks were made on the above by substituting 100 cc. of water for the starch in two flasks, incubating one

of these for the same length of time and placing the other in boiling water for 10 minutes to kill the enzyme. The diastase activity determinations therefore included the following in each case: one flask containing juice plus starch, incubated 12 hours; one flask containing juice plus water, incubated 12 hours; one flask containing juice plus water, boiled at once to kill the enzyme. The cupric-reducing power of the solution in the first flask less that of the solution in the third flask is considered to represent the activity of the enzyme.

Some preliminary work seemed to indicate the presence of an activating agent in the potato juice, in the absence of which the enzyme possessed little or no activity. To determine this point, some experiments were carried out according to the following procedure: The enzyme, which was precipitated from about 200 cc. of potato juice by the addition of three volumes of 95 per cent alcohol, was collected on filter-paper and dried, and then dissolved in 100 cc. of water and filtered, after which it was ready for use. Another 200-cc. sample of the juice was boiled, killing the enzyme and precipitating the proteins. This was also filtered before using.

In making the determinations, flasks were prepared as below. The same amounts of diastase solution and boiled juice were used in each instance, water being added to make the volume the same in all cases. Two per cent of toluol was used as a preservative.

One flask containing starch plus enzyme.
One flask containing starch plus enzyme plus boiled juice.
  These were incubated for varying periods of 12 to 24 hours at 45° C.
One flask containing enzyme plus boiled juice.
  This was boiled at the beginning of the experiment to kill the enzyme.

After incubation the cupric-reducing powers of the solutions were determined and these accepted as an index of the diastatic activity.

*Extraction of sugars.*—For the sugar determinations, 50 gms. of the green tubers (from the same lot used in the enzyme activity determinations) were weighed out and immediately sliced

into thin slices which were dropped into about 200 cc. of boiling 95 per cent alcohol containing 1 per cent of strong ammonia. After boiling for 10 or 15 minutes, this material was transferred, as nearly quantitatively as possible, to a Soxhlet extraction apparatus and extracted for 18 to 24 hours. After washing out the extraction apparatus with alcohol, this extract, which usually amounted to 400–500 cc., was then evaporated *in vacuo* at a temperature of 35–40° C., leaving finally a volume of 40–50 cc. This extract always contained a certain amount of loose starch, which was removed by centrifuging, after which the extract was made up to 200 cc. with doubly distilled water. When the analyses could not be made at once, 2 per cent toluol was added as a preservative.

This method of extraction and evaporation *in vacuo* is essentially that described by Davis, Daish, and Sawyer ('16) and is quite effective in removing the sugars. The necessity for transferring the material from one vessel to another several times during the process introduces the possibility of error, but with careful handling this is small.

*Estimation of reducing sugars.*—In making the determinations of reducing sugars, the method described by Shaffer ('14) was employed, with slight modifications. Forty cc. of the sugar solution were used instead of the 10 cc. recommended. This was done on account of the dilution, which made duplicates difficult to obtain when only 10 cc. were used. Five cc. of water and 5 cc. of colloidal iron brought the total to 50 cc., instead of 40 cc. as in the original method used by Shaffer. After removing the iron by centrifuging and filtering, two 15-cc. samples of the filtrate were used for the determinations, each sample corresponding to 12 cc. of the original sugar solution. The dissolved cuprous oxide was titrated with $\frac{N}{20}$ potassium permanganate, and duplicates checked within .1 or .2 cc. of permanganate.

*Determination of sucrose.*—For the determination of sucrose, the method used was the same up to the point where the two 15-cc. samples were measured out. Then 1.5 gms. (10 per cent) solid citric acid were added to each sample, as recommended by Davis and Daish ('13) for the inversion of sucrose. The samples

plus the citric acid were introduced directly into 50-cc. centrifuge tubes and placed in a boiling water bath for 10 minutes, after which the acid was neutralized to phenolphthalein with normal sodium hydroxide, Fehling's solution added, and the balance of the process completed by the Shaffer method. The sucrose was then determined as glucose by the increase in the cupric-reducing power of the solution, due to inversion of the sucrose.

*Starch determinations.*—The material from which the sugars had been extracted was used in the starch determinations. This was usually sufficiently dried by standing in the air for a few hours so that it could be ground up finely in a mortar. It was then carefully weighed and exactly ⅕ of it measured out, made into a paste by adding a small amount of water, and the paste poured into 400 or 500 cc. of boiling distilled water. Then the starch which had been centrifuged out of the sugar solution was suspended in 100 cc. of distilled water, and 20 cc. ($\frac{1}{5}$) pipetted out and added to the other. The whole was then gelatinized by boiling under a reflux condenser for two hours, cooled, and made up to one liter with distilled water, 2 per cent toluol being added to prevent the growth of microörganisms. One-tenth gm. Taka-diastase was then added to 100 cc. of the solution, which was well shaken during sampling, and this was incubated at 45° C. for 24 hours. Under these conditions, according to Davis and Daish ('14), hydrolysis of the starch is complete, it having been broken down into glucose and maltose, which exist in a definite ratio in the solution. These sugars were then determined as glucose. The value found, of course, is not the true starch value, but may be used as a basis for comparison, which is all that is demanded in this work.

## Results and Discussion

*Diastase activity.*—The results of the diastase activity determinations are given below (table 1). These results are, of course, comparative only. The figures in the first three columns represent the number of cubic centimeters of potassium permanganate used in titrating the dissolved cuprous oxide in one sample, while the relative enzyme activity (the difference between the figures in the first and third columns) is given in the

fourth column. The latter is expressed in terms of 100, which is assumed to be the value of the greatest activity observed.

TABLE I

DIASTASE ACTIVITY IN RELATION TO STAGE OF DEVELOPMENT OF POTATO TUBERS*

| Stage of development (diameter in cms.) | No. cc. $\frac{N}{20}$ $KMnO_4$ used in titrating $Cu_2O$ | | | Relative diastatic activity |
|---|---|---|---|---|
| | Juice+starch (incubated) | Juice+water (incubated) | Juice+water (boiled) | |
| 1 | 10.7 | Lost | 8.0 | 66 |
| 2 | 13.0 | 11.4 | 10.1 | 71 |
| 3 | 12.8 | 10.0 | 8.7 | 100 |
| 4 | 9.9 | 7.1 | 6.5 | 83 |
| 5 | 6.2 | 3.5 | 2.2 | 98 |
| 6 | 5.7 | 2.9 | 1.7 | 98 |
| Seed potato | 11.0 | 8.4 | 7.8 | 78 |
| Seed potato | 8.2 | 6.6 | 6.5 | 41 |
| Seed potato | 9.6 | 7.3 | 7.1 | 61 |

* In the growing potatoes, not less than 500 gms. of the tubers were used, in each case, as the source of the juice.

It appears from these figures that advance in stage of development is accompanied by an increase in diastatic activity. The author is inclined to believe that the high value obtained for tubers 3 cm. in diameter is perhaps abnormal and without particular significance.

The diastatic activity of three samples of seed potatoes is also given for purposes of comparison. The first two were taken from the lot that was used for seed, while the third represents a different variety. They had been kept in ordinary storage up to the time the analyses were made. It will be observed that their diastatic activity is considerably lower than that of most of the growing tubers. It is rather surprising that the two samples from the same lot of stored tubers should possess such widely different diastatic activities as 78 and 41.

The second column in table I is included to show the increase in reducing sugars which occurs when the juice alone (diluted

with water) is incubated. If the figures in the third column, which represent the diluted juice boiled at the beginning of the experiment to kill the enzyme, are compared with those in the second, it will be seen that there is a considerable increase in reducing sugars. This is probably due to conversion, by the enzyme, of dextrins in the juice, to reducing sugars. The differences in this respect are less in the case of the seed potatoes, which corresponds with their lower enzyme activity.

The activating effect of boiled potato juice upon the diastase of potatoes has been observed by Doby ('14). The table given below (table II) shows some results obtained by the writer on this point. The figures represent cubic centimeters of potassium permanganate used in titrating the dissolved cuprous oxide. The "enzyme" used is that obtained by precipitation with alcohol, as has already been described.

TABLE II

EFFECT OF BOILED JUICE UPON THE ACTIVITY OF POTATO DIASTASE

| Sample | No. cc. $\frac{N}{20}$ $KMnO_4$ used in titrating $Cu_2O$ | | | Difference due to enzyme in presence of boiled juice |
|---|---|---|---|---|
| | Enzyme+starch | Enzyme+starch+boiled filtered juice | Boiled filtered juice | |
| 1 | 0 | 4.1 | 2.8 | 1.3 |
| 2 | 0 | 5.6 | 4.3 | 1.3 |
| 3 | 0 | 2.2 | .6 | 1.6 |
| 4 | .4 | 26.0 | 24.6 | 1.4 |
| 5 | .3 | 5.9 | 4.9 | 1.0 |

From the figures given, it seems that the activity of diastase of potatoes depends upon some substance contained in the juice, but not precipitated by alcohol. It is well known that a number of substances have an activating effect upon diastase, but it has been generally considered that the enzyme when alone would act upon starch, and as far as the author is aware, no one has before presented evidence to show that this is not always the case.

In the first tests made, no activity whatever was observed when the enzyme alone was added to the starch solution, but in

the last two a very small amount of starch was hydrolyzed after 24 hours. While the latter results must be considered positive, the figures are so small as to be almost within the range of experimental error.

Since the activity of diastase is accelerated by small amounts of acids, it was thought that the activating effect of the boiled juice might possibly be due to its hydrogen ion concentration. Accordingly, determinations were made in two cases which gave an H ion concentration of $P_H$ 5.7 and $P_H$ 6.2. This very slight acidity could hardly influence the activity of the enzyme. However, a solution consisting of mono- and dipotassium phosphate having the same $P_H$ value was substituted for the boiled juice in each case, but failed to have any activating effect.

Another point of some interest in regard to diastase activity was incidentally observed in the course of these experiments, that is, the direct effect of the time factor upon diastatic activity of potato juice. Doby and Bodnar ('15) report that when the juice was preserved with toluol and allowed to stand in the dark at 8–10° C. for 24 hours or longer, its diastatic activity increased. As stated, the results reported here were obtained incidentally, without any effort to provide Doby and Bodnar's conditions, and are given for what they are worth (table III). The juice used was preserved with 2 per cent toluol and stood at room temperature in partial light.

TABLE III

EFFECT OF THE TIME FACTOR UPON THE DIASTATIC ACTIVITY OF POTATO JUICE

| Sample | Relative diastatic activity | |
|---|---|---|
| | Fresh juice | After standing 24 hrs. |
| 1 | 98 | 66 |
| 2 | 120 | 59 |

These results indicate that under the conditions which obtained in the experiment there is a marked decrease in the diastatic activity of the potato juice on standing 24 hours. This is directly opposed to the conclusions of Doby and Bodnar, who,

however, it must be remembered, kept the juice under different conditions. In view of the results given here, it would seem desirable to repeat the work of the latter authors, employing the conditions under which their results were obtained.

*Carbohydrate content of potatoes.*—The carbohydrate content of the tubers used in this work is given in table IV. The figures given show the amounts of reducing sugars, sucrose, and starch, all determined as glucose, present in 50 gms. (green weight) of the freshly dug tubers at each stage of growth. No analyses were made of tubers 1 cm. in diameter, due to the fact, as stated before, that while enough of the potatoes of this size were available for the diastase activity determinations, there was not sufficient quantity to make also the sugar and starch determinations.

The results given in table IV show a gradual decrease, with advance in development, in the amount of reducing sugar present, which is accompanied up to a certain point by a corresponding increase in the amount of sucrose. The latter falls off rapidly, however, as the tuber approaches maturity. This is in agreement with the limited data of Hungerbühler ('86), who also found a decrease in the reducing sugars and an increase in invert sugar, with advance in maturity. The results of Schulze and Seliwanoff ('88), who found sucrose to be abundant in the immature tubers, are also confirmed.

TABLE IV

CARBOHYDRATE CONTENT OF POTATO TUBERS AT DIFFERENT STAGES OF GROWTH

| Stage of growth (diameter in cms.) | No. gms. carbohydrate, determined as glucose, in 50 gms. green tubers | | |
|---|---|---|---|
| | Reducing sugar | Sucrose | Starch |
| 2 | .3854 | .4217 | 6.963 |
| 3 | .3307 | .4395 | 6.039 |
| 4 | .2388 | .6358 | 6.236 |
| 5 | .0774 | .6194 | 7.494 |
| 6 | .0127 | .0924 | 7.632 |
| Seed potato | .1360 | .0722 | 7.065 |
| Seed potato | .1857 | .0675 | 8.650 |

The results obtained may also be considered to agree with those found by Keitt ('11) for the sweet-potato. The latter found the total sugars in the sweet-potato tuber to decrease toward maturity, and, from the figures given above, the same tendency appears to exist in the Irish potato. In the case of the seed potatoes, the reducing sugars are present in much larger quantity than in the larger sizes of the growing tubers, while the sucrose is lower than the lowest value obtained in the case of the growing potatoes.

It is somewhat difficult to interpret the sugar relations of the potato tuber, as shown by the figures given, but the following is a possible explanation: Sugars in plants are translocated chiefly in the form of the hexoses, glucose and levulose. When photosynthesis and growth are proceeding at a rapid rate in the early stages of development and when diastase activity is not so great as later on, it would be expected that the translocated sugars (reducing sugars), would be present in the potato tuber in comparatively large amounts. Diastase, acting as a synthesizing agent, would gradually convert the glucose into the storage product, starch, but this alone might not suffice to dispose of all the reducing sugars accumulating in the tuber, so the invertase present may also be conceived to act as a synthesizing agent and transform some of it into a temporary storage product, sucrose, which accumulates up to a certain point. Towards maturity, as the translocation of hexoses becomes less rapid and as the glucose which reaches the tuber is more rapidly converted into starch by the more active diastase, less reducing sugar is found present. As the reducing sugar decreases, some of the sucrose is hydrolyzed to hexose by the invertase, possibly in connection with the general matter of equilibrium relations, and thus a decrease in the sucrose is brought about. Under the influence of the low temperatures which prevail in storage, the starch may be partially hydrolyzed to reducing sugar, some of which may again be built up into sucrose, thus causing an accumulation in the tubers, such as has been observed by a number of investigators.

An examination of the values obtained in the starch determinations shows that the percentage of this carbohydrate increases gradually as the tuber enlarges in size. The relatively high

value found in the case of tubers 2 cm. in diameter does not follow the general trend, and remains unexplained. The two samples of seed potatoes exhibit a rather wide difference in starch content, which, the author believes, is perhaps due to varietal differences or to a difference in the conditions under which the potatoes were kept. The conditions under which the tubers were grown may also have exerted some influence in this respect.

## SUMMARY

A review of the literature, bearing directly or indirectly upon the subject of this paper, is presented.

The methods of experimentation are described. They include the determination of diastase activity of potato tubers at various stages of development by adding fresh potato juice to a solution of soluble starch; the extraction and estimation of reducing sugars and sucrose by the comparatively new method of Davis, Daish, and Sawyer; and the estimation of starch by means of Taka-diastase.

The following results were obtained:

Diastase activity and starch content were found to increase with advance in the development of the tubers.

In general, the increase in enzyme activity and starch content of growing potatoes was accompanied by a decrease in the total sugars present.

When the juice of potatoes was preserved with toluol and kept for 24 hours at room temperature, its diastatic activity was found to decrease quite markedly.

Evidence was obtained which indicates that a co-enzyme is necessary in the hydrolysis of starch by potato diastase. The activating agent exists in the juice, and is not destroyed by boiling, nor is it precipitated by alcohol.

It is the opinion of the author that if a number of analyses were made along the lines described and the average of these taken the comprehensive results thus obtained would clear up the points which, in this report, appear somewhat doubtful, and would make it possible to draw definite conclusions.

The writer wishes to acknowledge his indebtedness to Dr.

B. M. Duggar for suggesting the problem and for his kindly advice and criticism during its prosecution. Thanks are also due Dr. George T. Moore for the privileges of the library and laboratory of the Missouri Botanical Garden, and to Mr. Henry Schmitz and Mr. Alexander Lurie for assistance in connection with the literature.

*Graduate Laboratory, Missouri Botanical Garden.*

## Bibliography

Appleman, C. O. ('11). Physiological behavior of enzymes and carbohydrate transformations in after-ripening of the potato tuber. Bot. Gaz. 52: 306–315. 1911.

———, ('16). Biochemical and physiological study of the rest period in the tubers of Solanum tuberosum. *Ibid.* 61: 265–294. *f. 1–2.* 1916.

Baranetzky, J. ('78). Die Stärkeumbildenden Fermente. 64 pp. *1 pl.* Leipzig, 1878.

Bonns, W. W. ('18). Etherization of tissues and its effect on enzyme activity. Ann. Mo. Bot. Gard. 5: 225–299. 1918.

Bradley, H. C., and Kellersberger, E. ('12). The problem of enzyme synthesis. III. Diastase and starch of plant tissues. Jour. Biol. Chem. 13: 425–429. 1912.

Brasse, L. ('84). Sur la présence de l'amylase dans les feuilles. Compt. Rend. Acad. Paris 99: 878–879. 1884.

Brown, H. T., and Morris, G. H. ('90). Researches on the germination of some of the Gramineae. Jour. Chem. Soc., Trans. 57: 458–528. 1890.

———, ———, ('93). A contribution to the chemistry and physiology of foliage leaves. *Ibid.* 63: 604–677. 1893.

Butler, O. ('13). A note on the significance of sugar in the tubers of Solanum tuberosum. Torr. Bot. Club, Bul. 40: 110–118. 1913.

Czapek, F. ('13). Biochemie der Pflanzen. 1913. [See pp. 470–471.]

Davis, A. R. ('15). Enzyme action in marine algae. Ann. Mo. Bot. Gard. 2: 771–836. 1915.

Davis, W. A., and Daish, A. J. ('13). A study of the methods of estimation of carbohydrates, especially in plant extracts. A new method for the estimation of maltose in the presence of other sugars. Jour. Agr. Sci. 5: 437–468. 1913.

———, ———,('14). Methods of estimating carbohydrates. II. The estimation of starch in plant material. The use of Taka-diastase. *Ibid.* 6: 152–168. 1914.

———, ———, and Sawyer, G. C. ('16). Studies of the formation and translocation of carbohydrates in plants. I. The carbohydrates of the mangold leaf. *Ibid.* 7: 255–326. 1916.

———, and Sawyer, G. C. ('16). *Ibid.* II. The carbohydrates of the leaf and leaf stalks of the potato. The mechanism of the degradation of starch in the leaf. *Ibid.* 7: 352–384. 1916.

Doby, G. ('14). Ueber Pflanzenenzyme. II. Die Amylase der Kartoffelknolle. Biochem. Zeitschr. **67**: 166–181. 1914.

———, und Bodnar, J. ('15). *Ibid.* III. Pathologische Veranderungen der Kartoffelamylase. *Ibid.* **68**: 191–205. 1915.

Duggar, B. M., and Davis, A. R. ('14). Enzyme action in Fucus vesiculosus L. Ann. Mo. Bot. Gard. **1**: 419–426. 1914.

Effront, J. ('02). Enzymes and their applications. 1902. [Translation by Prescott.]

Green, J. R. ('97). On the action of light on diastase, and its biological significance. Roy. Soc. London, Phil. Trans. B **188**: 167–190. 1897.

———, ('99). The soluble ferments and fermentation. 1899.

Haas, P., and Hill, T. G. ('17). An introduction to the chemistry of plant products. 1917. [See p. 369.]

Hasselbring, H. ('18). Behavior of sweet potatoes in the ground. Jour. Agr. Res. **12**: 9–17. 1918.

———, and Hawkins, L. A. ('15). Physiological changes in sweet potatoes during storage. *Ibid.* **3**: 331–342. 1915.

———, ———, ('15a). Carbohydrate transformations in sweet potatoes. *Ibid.* **5**: 543–560. 1915.

Howard, W. L. ('15). An experimental study of the rest period in plants. Physiological changes accompanying breaking of the rest period. Mo. Agr. Exp. Sta., Res. Bul. **21**: 1–72. 1915.

Hungerbühler, F. ('86). Landw. Versuchsstat. **32**: 381. 1886. [Cited by Czapek, '13, p. 470.]

Jörgensen, I., and Stiles, W. ('17). Carbon assimilation. A review of the recent work on the pigments of the green leaf and the processes connected with them. New Phytol. Reprint No. 10: 1–180. 1917. [Published first in New Phytol. **14**, **15**, and **16**.]

Keitt, T. E. ('11). The formation of sugars and starch in the sweet potato. S. C. Agr. Exp. Sta., Bul. **156**: 1–14. 1911.

Meyer, A. ('85). Ueber die Assimilationsproducte der Laubblätter angiospermer Pflanzen. Bot. Zeit. **43**: 417–423, 433–440, 449–457, 465–472, 481–491, 497–507. 1885.

Müller-Thurgau, H. ('82). Ueber Zukeranhäufung in Pflanzentheilen in Folge niederer Temperatur. Landw. Jahrb. **11**: 751–828. 1882.

———, ('85). Beiträge zur Erklärung der Ruheperioden der Pflanzen. *Ibid.* **14**: 851–907. 1885.

Norris, R. V. ('14). The hydrolysis of glycogen by diastatic enzymes. III. Factors influencing the end point of the hydrolysis. Biochem. Jour. **3**: 421–433. 1914.

Payen and Persoz ('33). Mémoire sur la diastase, les principaux produits de ses réactions, et leurs applications aux arts industriels. Ann. de Chim. et de Phys. **53**: 73–92. 1833.

Prunet, A. ('92). Sur le mécanisme de la dissolution de l'amidon dans la plante. Compt. Rend. Acad. Paris **115**: 751–754. 1892.

Schimper, A. F. W. ('85). Ueber Bildung und Wanderung der Kohlehydrate in den Laubblättern. Bot. Zeit. 43: 737–743, 753–763, 769–787. 1885.

Schulze, E., und Seliwanoff. ('88). Landw. Versuchsstat. 34: 403. 1888. [Cited by Czapek, '13, p. 470.]

Shaffer, P. A. ('14). On the determination of sugar in blood. Jour. Biol. Chem. 19: 285–295. 1914.

Thatcher, R. W. ('15). Enzymes of apples and their relation to the ripening process. Jour. Agr. Res. 5: 103–116. 1915.

Waugh, F. A. ('98). On the artificial use of enzymes in germination. Vt. Agr. Exp. Sta., Bul. 11: 290–295. 1898.

Wortmann, J. ('90). Ueber den Nachweis, das Vorkommen und die Bedeutung des diastatischen Enzyms in den Pflanzen. Bot. Zeit. 48: 581–594, 597–607, 617–627, 633–654, 657–669. 1890.